



IMMUNOLOGICAL STUDIES ON HUMAN CHORIONIC GONADOTROPIN

ABSTRACT

By

AMITA GUPTA

DEPARTMENT OF BIOCHEMISTRY

JAWAHARLAL NEHRU MEDICAL COLLEGE

ALIGARH MUSLIM UNIVERSITY

ALIGARH-202 002

3604

A Thesis Submitted in Fulfilment of the Requirements for the
Degree of Doctor of Philosophy in Biochemistry in the
Faculty of Medicine of the Aligarh Muslim University

ALIGARH

1988

A B S T R A C T

The concept of vaccine against pregnancy is an attractive approach of fertility control and one which has received increasing attention in the last decade. The luteinizing hormone (LH) and chorionic gonadotropin (hCG) exercise a critical role in the regulation of fertility of primates. The former is important in ovulation and steroidogenesis but the latter for rescue of corpus luteum and maintenance of steroidogenesis upto the time of placental shift. HCG is produced by the trophoblast cells of placenta and its concentration increases rapidly post-fertilization. HCG is a 40 kD M.W. glycoprotein hormone composed of two non-identical subunits designated as alpha (α) and beta (β). HCG and other related pituitary hormones such as LH, FSH and TSH consist of nearly identical alpha chains, although, their beta chains show varying degree of sequential homology. Therefore, the antibodies capable of neutralizing the biological activity of hCG may also cross react with LH. However, beta-subunit of LH lacks the β -hCG specific C-terminal segment of 32 amino acid residues.

In the present investigation, hCG was purified from a commercial preparation by gel filtration and its purity was checked by polyacrylamide gel electrophoresis. The SDS-PAGE revealed that the hormone comprised two dissimilar subunits, α and β . The UV irradiation of hCG at 254 nm was found to cause a dose-dependent loss in intrinsic fluorescence intensity and

UV absorbance at 275 nm. Six lysine residues were modified by trinitrophenylation in native hCG whereas in irradiated hCG only 5 residues were modified, indicating destruction of one lysine residue and/or structural change as a consequence of irradiation. The observation is substantiated by the proteolysis of native and irradiated hCG. In the native hCG sites of tryptic attack were found to be buried but in the irradiated molecules these were accessible to tryptic attack due to change in structure of the molecule. However, as the radiation dose is increased, the susceptibility of hCG to trypsin decreased. This might be due to either the destruction of amino acids that are available for digestion or there is a partial change in structure of irradiated hCG molecule in a way that amino acids are no more accessible to tryptic digestion. Similar results were obtained on urea treatment of irradiated hCG.

Precipitating antibodies against native and irradiated hCG were successfully induced in rabbits. Antisera against irradiated hCG showed increased antibody level with period of immunization. The titer was found to be 1:100, 1:400 and 1:1600 after second, third and fourth injections respectively. Immunohistological studies on the effect of native hCG on testes showed increased spermatogenesis while with irradiated sample, the maturation of sperms was arrested. These studies on native and irradiated hCG is indicative of the belief that immunogenicity and biological activity of molecules are associated

with their native structure. UV irradiation has affected the native structure of hCG with decreased biological activity.

The evaluation of binding of psoralen with hCG was ascertained by fluorescence quenching technique and UV absorption. The hCG-psoralen complex exhibited increased absorbance over the entire spectral range of 200 nm to 370 nm. The fluorescence emission spectra of the complex was unchanged but showed a decrease in fluorescence intensity. The Scatchard plot analysis of the interaction indicates the involvement of four binding sites ($r = 3.9$) with an apparent association constant, $K_a = 0.795 \times 10^5 \text{ M}^{-1}$. As a result of interaction between hCG and psoralen, an increased absorption at 275 nm was observed, without a change in UV absorption spectra. When the samples were subjected to gel filtration on Sephadex G-100 column. The binding between these two interacting molecules was further reiterated by the capacity of psoralen to compete in hCG-anti-hCG antibody system. Appreciable inhibition in immunoreactivity in the presence of psoralen was observed. The results also indicate a possible correlation between the binding site of anti-hCG antibody and psoralen on the hCG molecule.

The complex of psoralen and hCG was more immunogenic than the free hormone and antibody titer of 1:1920, 1:15360 and 1:491520 was acquired after third, fourth and fifth injections respectively. In order to rule out the possibility of unspecific binding, inhibition ELISA was performed. The results suggest

that after binding with psoralen, hCG became more antigenic.

The binding of psoralen to subunits of hCG was also investigated. The alpha-psoralen and beta-psoralen complex exhibited no change in their relative excitation and emission wavelengths but showed a significant quenching in intensity. Similar results were obtained with ultraviolet spectral studies and gel filtration experiments.

Antibodies were induced in rabbits against complexes of psoralen with alpha and beta-subunits of hCG. In contrast to hCG-psoralen complex, the subunit complexes were less antigenic as compared to free subunits. It appears that the binding sites of psoralen on the subunits of hCG and epitope are similar in structure. These two entity might be close to one another.

Three peptides (PI, PII and PIII) of β -subunit of hCG were separated and isolated by gel filtration on Sephadex G-25 after hydrolysis by chymotrypsin of carboxymethylated β hCG. These peptides were purified on Sephadex G-50 and used as inhibitors in anti-hCG and anti- β hCG antibodies. Maximum inhibition was found with PI whereas no inhibition obtained with PIII.

Anti-PI IgG was isolated from anti-hCG serum by affinity chromatography using a column of Sepharose 4B coupled with PI. The affinity purified anti-PI IgG was checked for antibody

activity with hCG, β hCG, α hCG, PI, PII and PIII in inhibition ELISA. No reactivity was observed with α hCG, PI and PII.

Antibodies were only reactive with hCG and β hCG indicating that epitopes on PI are part of hCG and β hCG but not of α hCG.



IMMUNOLOGICAL STUDIES ON HUMAN CHORIONIC GONADOTROPIN

By

AMITA GUPTA

DEPARTMENT OF BIOCHEMISTRY

JAWAHARLAL NEHRU MEDICAL COLLEGE

ALIGARH MUSLIM UNIVERSITY

ALIGARH-202 002

Date _____

Approved :

Rashid Ali,

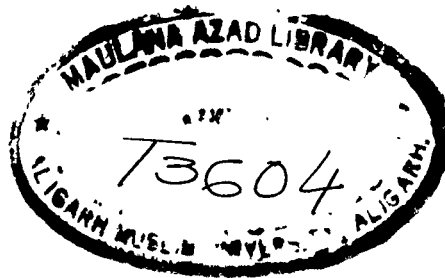
Supervisor

A Thesis Submitted in Fulfilment of the Requirements for the
Degree of Doctor of Philosophy in Biochemistry in the
Faculty of Medicine of the Aligarh Muslim University

ALIGARH

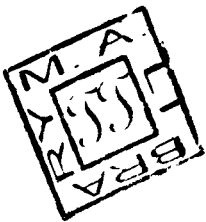
1988

2
CHECKED-2002



31 MAY 1989

THESIS SECTION



T3604

C E R T I F I C A T E

I certify that the work presented in the following pages has been carried out by Mrs. Amita Gupta and is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.



(Rashid Ali)

Professor and Chairman
Department of Biochemistry
Jawaharlal Nehru Medical College
Aligarh Muslim University
ALIGARH - 202 001

ACKNOWLEDGEMENTS

I deem it as a singular honour in placing on record my deep debt of gratitude to my supervisor, Prof. Rashid Ali, Chairman, Department of Biochemistry, J.N. Medical College, Aligarh Muslim University, Aligarh for an excellent guidance, continued encouragement, valuable discussions and devotion of his precious time towards this investigation.

I express my thanks to Prof. A. Salahuddin for encouragement and valuable suggestions.

I appreciate the cooperation and valuable suggestions of my colleagues (Drs.) Rashid Hasan, Israr A. Khan, Asif Ali, Ashok Kumar, Manzoor A. Shah, Manju Gupta, (Messers) Najmul Islam, Naushad Ali, Khurseed Alam, Mrs. Vandna Sehgal and Miss. Jahan Ara.

I acknowledge my thanks to my husband Capt. R.K. Gupta who enhanced my spirit and supported my enthusiasm. My little daughter Ritika's parting patience also allowed me to concentrate to work on this project.

The excellent typing of the script by Mr. Mazahir Husain is much appreciated.

Financial assistance by Council of Scientific and Industrial Research is gratefully acknowledged.

Lastly I express my deepest sense of gratitude to my parents who were always with me mentally and emotionally to give moral support.

Amita Gupta
(AMITA GUPTA)

C O N T E N T S

			Page
ABSTRACT	1
LIST OF FIGURES	6
LIST OF TABLES	10
INTRODUCTION	11
EXPERIMENTAL	40
RESULTS	62
DISCUSSION	124
REFERENCES	131

A B S T R A C T

The concept of vaccine against pregnancy is an attractive approach of fertility control and one which has received increasing attention in the last decade. The luteinizing hormone (LH) and chorionic gonadotropin (hCG) exercise a critical role in the regulation of fertility of primates. The former is important in ovulation and steroidogenesis but the latter for rescue of corpus luteum and maintenance of steroidogenesis upto the time of placental shift. HCG is produced by the trophoblast cells of placenta and its concentration increases rapidly post-fertilization. HCG is a 40 kD M.W. glycoprotein hormone composed of two non-identical subunits designated as alpha (α) and beta (β). HCG and other related pituitary hormones such as LH, FSH and TSH consist of nearly identical alpha chains, although, their beta chains show varying degree of sequential homology. Therefore, the antibodies capable of neutralizing the biological activity of hCG may also cross react with LH. However, beta-subunit of LH lacks the β -hCG specific C-terminal segment of 32 amino acid residues.

In the present investigation, hCG was purified from a commercial preparation by gel filtration and its purity was checked by polyacrylamide gel electrophoresis. The SDS-PAGE revealed that the hormone comprised two dissimilar subunits, α and β . The UV irradiation of hCG at 254 nm was found to cause a dose-dependent loss in intrinsic fluorescence intensity and

UV absorbance at 275 nm. Six lysine residues were modified by trinitrophenylation in native hCG whereas in irradiated hCG only 5 residues were modified, indicating destruction of one lysine residue and/or structural change as a consequence of irradiation. The observation is substantiated by the proteolysis of native and irradiated hCG. In the native hCG sites of tryptic attack were found to be buried but in the irradiated molecules these were accessible to tryptic attack due to change in structure of the molecule. However, as the radiation dose is increased, the susceptibility of hCG to trypsin decreased. This might be due to either the destruction of amino acids that are available for digestion or there is a partial change in structure of irradiated hCG molecule in a way that amino acids are no more accessible to tryptic digestion. Similar results were obtained on urea treatment of irradiated hCG.

Precipitating antibodies against native and irradiated hCG were successfully induced in rabbits. Antisera against irradiated hCG showed increased antibody level with period of immunization. The titer was found to be 1:100, 1:400 and 1:1600 after second, third and fourth injections respectively. Immunohistological studies on the effect of native hCG on testes showed increased spermatogenesis while with irradiated sample, the maturation of sperms was arrested. These studies on native and irradiated hCG is indicative of the belief that immunogenicity and biological activity of molecules are associated

with their native structure. UV irradiation has affected the native structure of hCG with decreased biological activity.

The evaluation of binding of psoralen with hCG was ascertained by fluorescence quenching technique and UV absorption. The hCG-psoralen complex exhibited increased absorbance over the entire spectral range of 200 nm to 370 nm. The fluorescence emission spectra of the complex was unchanged but showed a decrease in fluorescence intensity. The Scatchard plot analysis of the interaction indicates the involvement of four binding sites ($r = 3.9$) with an apparent association constant, $K_a = 0.795 \times 10^5 \text{ M}^{-1}$. As a result of interaction between hCG and psoralen, an increased absorption at 275 nm was observed, without a change in UV absorption spectra. When the samples were subjected to gel filtration on Sephadex G-100 column. The binding between these two interacting molecules was further reiterated by the capacity of psoralen to compete in hCG-anti-hCG antibody system. Appreciable inhibition in immunoreactivity in the presence of psoralen was observed. The results also indicate a possible correlation between the binding site of anti-hCG antibody and psoralen on the hCG molecule.

The complex of psoralen and hCG was more immunogenic than the free hormone and antibody titer of 1:1920, 1:15360 and 1:491520 was acquired after third, fourth and fifth injections respectively. In order to rule out the possibility of unspecific binding, inhibition ELISA was performed. The results suggest

that after binding with psoralen, hCG became more antigenic.

The binding of psoralen to subunits of hCG was also investigated. The alpha-psoralen and beta-psoralen complex exhibited no change in their relative excitation and emission wavelengths but showed a significant quenching in intensity. Similar results were obtained with ultraviolet spectral studies and gel filtration experiments.

Antibodies were induced in rabbits against complexes of psoralen with alpha and beta-subunits of hCG. In contrast to hCG-psoralen complex, the subunit complexes were less antigenic as compared to free subunits. It appears that the binding sites of psoralen on the subunits of hCG and epitope are similar in structure. These two entity might be close to one another.

Three peptides (PI, PII and PIII) of β -subunit of hCG were separated and isolated by gel filtration on Sephadex G-25 after hydrolysis by chymotrypsin of carboxymethylated β hCG. These peptides were purified on Sephadex G-50 and used as inhibitors in anti-hCG and anti- β hCG antibodies. Maximum inhibition was found with PI whereas no inhibition obtained with PIII.

Anti-PI IgG was isolated from anti-hCG serum by affinity chromatography using a column of Sepharose 4B coupled with PI. The affinity purified anti-PI IgG was checked for antibody

activity with hCG, β hCG, ahCG, PI, PII and PIII in inhibition ELISA. No reactivity was observed with ahCG, PI and PII.

Antibodies were only reactive with hCG and β hCG indicating that epitopes on PI are part of hCG and β hCG but not of ahCG.

LIST OF FIGURES

	Page
Fig.1(a) . The amino acid sequence of alpha-subunit of hCG.	15
Fig.1(b). The amino acid sequence of beta-subunit of hCG.	16
Fig.2. Structures for oligosaccharides chains of hCG.	18
Fig.3. Separation of hCG-subunits by DEAE cellulose chromatography.	45
Fig.4. Isolation of serum IgG by DEAE cellulose chromatography.	58
Fig.5. Gel filtration of commercial hCG on Sephadex G-100 column.	63
Fig.6. Elution profile of purified hCG on Sephadex G-100 column.	64
Fig.7. Polyacrylamide gel electrophoretic pattern of commercial and purified hCG.	65
Fig.8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of hCG.	66
Fig.9. Ultraviolet and fluorescence spectra of native hCG.	68
Fig.10. Fluorescence difference spectra of photo-inactivated hCG.	69

Fig.11.	Ultraviolet spectra of photoinactivated hCG.	70
Fig.12.	Kinetics of modification of lysine residues of native and irradiated hCG with 2,4,6-trinitrobenzene sulfonic acid (TNBS).	73
Fig.13.	Effect of ultraviolet irradiation on tryptic-digestibility of hCG.	74
Fig.14.	Effect of irradiation on reducing sugars.	75
Fig.15.	Urea denaturation profiles of native and irradiated hCG.	77
Fig.16.	Antibodies against hCG by CIE.	78
Fig.17.	Direct binding of hCG by induced antibodies against irradiated hCG.	80
Fig.18.	Histology of rat testes after immunization.	81
Fig.19.	Ultraviolet spectra of dark interaction between psoralen and hCG.	83
Fig.20.	Fluorescence spectra of hCG-psoralen complex in dark.	84
Fig.21.	Scatchard plot for binding of psoralen to hCG in dark.	86
Fig.22.	Plot of binding parameters of hCG-psoralen complex.	88
Fig.23.	Plot of binding of psoralen to hCG in terms of percent quenching.	89
Fig.24.	Hydrodynamic behaviour of hCG and hCG-psoralen complex on Sephadex G-100 column.	90

Fig.25.	Effect of psoralen on hCG-anti-hCG antibody binding capacity.	91
Fig.26.	Binding of hCG by induced antibodies against hCG-psoralen complex.	93
Fig.27.	A comparative study of titers of induced antibodies against hCG and hCG-psoralen complex.	94
Fig.28.	Antibody activity of anti-hCG-psoralen IgG.	95
Fig.29.	Inhibition ELISA for anti-hCG-antibodies.	96
Fig.30.	Inhibition ELISA with anti-hCG-psoralen antibodies.	97
Fig.31.	Inhibition ELISA with anti-hCG-psoralen IgG.	99
Fig.32.	Ultraviolet spectra of UV-A irradiated hCG-psoralen complex.	100
Fig.33.	Effect of time of UV-A irradiation on binding capacity of psoralen to hCG.	102
Fig.34.	Effect of psoralen concentration upon binding to hCG in presence of UV-A light.	103
Fig.35.	Effect of urea on stability of hCG.	104
Fig.36.	Fluorescence spectra of dark interaction between alpha-subunit and psoralen.	106
Fig.37.	Fluorescence spectra of dark interaction between beta-subunit and psoralen.	107

Fig.38.	Ultraviolet spectra of psoralen bound alpha-subunit in dark.	108
Fig.39.	Ultraviolet spectra of psoralen bound beta-subunit in dark.	109
Fig.40.	Hydrodynamic behaviour of subunits of hCG and its complex with psoralen on Sephadex G-100 column.	111
Fig.41.	Binding of hCG by induced antibodies against alpha-psoralen complex and alpha subunit.	112
Fig.42.	Binding of hCG by induced antibodies against beta-psoralen complex and beta subunit.	113
Fig.43.	A comparative data of titers of induced antibodies against alpha-subunit and alpha-psoralen complex.	114
Fig.44.	A comparative data of titers of induced antibodies against beta-subunit and beta-psoralen complex.	115
Fig.45.	Gel filtration after hydrolysis of carboxy-methylated beta-subunit by chymotrypsin on Sephadex G-25 column.	117
Fig.46.	Purification of different peptides of beta-subunit on Sephadex G-50 column.	118
Fig.47.	Inhibitory capacity of peptides of beta-subunit against anti-hCG antibodies.	119
Fig.48.	Inhibitory capacity of peptides of beta-subunit against anti-beta hCG antibodies.	120
Fig.49.	Reactivity of affinity purified anti-peptide I IgG with different antigens.	122
Fig.50.	Reactivity of affinity purified anti-peptide I IgG with different peptides of beta-subunit.	123

LIST OF TABLES

	Page
Table I. Amino acid residues per molecule of hCG-subunits.	13
Table II. Carbohydrate composition of hCG and its subunits.	14
Table III. Ultraviolet absorption at 275 nm of native and irradiated hCG.	71

I N T R O D U C T I O N

An immunological approach to the prevention of pregnancy has received considerable attention during the last decade. The pituitary luteinizing hormone (LH) and human chorionic gonadotropin (hCG) exercise a critical role in the regulation of fertility of primates. The former is important for ovulation and steroidogenesis, but the latter for rescue of corpus luteum upto the time of placental shift. Antibodies capable of disrupting the action of hCG should prevent progesterone and estrogen production by the corpus luteum that is required by the endometrium for the support of blastocyst. Active and passive immunologic approaches have been proposed for the control of fertility by antibodies intercepting the action of one or both gonadotropins (Moudgal et al, 1978; Hulme et al, 1980; Tandon et al, 1981; Tandon et al, 1984).

HCG is a glycoprotein hormone synthesized and secreted by the syncytiotrophoblast cells of the placenta from early pregnancy until term. In the first trimester of pregnancy the synthesis of the hormone reaches its maximum level. HCG consists of two subunits, alpha (α) and beta (β) held together by non-covalent bonds. The alpha chain of hCG, is indistinguishable with other pituitary hormones as follicle stimulating hormone (FSH), thyrotropin (TSH) and luteinizing hormone (LH). With the exception of TSH, all of the hormones are involved in the biochemistry of reproduction. The β -subunits of all these hormones differs and

confers on the hormone its unique biological activity (Pierce et al, 1971a). The subunits of the hormone can be reassociated to restore most of the biological and immunological activities (Pierce et al, 1971b).

1. Chemistry of hCG

(i) Chemical composition

In hCG, the protein is covalently linked to carbohydrate. Preparations of high purity by biochemical and immunological criteria have enabled to obtain data on the amino acid and carbohydrate composition of hCG (Table 1). The most salient feature of the amino acid composition of hCG is a very high proline content. Quantitative estimation of carbohydrates from four laboratories are presented in Table 2.

(ii) Subunit structure

The non-identity of the subunits of hCG was first established by two groups of investigators, Canfield et al, (1970) and Swaminathan and Bahl (1970). Sequences for α -subunit and β -subunit of hCG have been elucidated (Fig.1). The α -subunit contains 92 amino acid residues and a total molecular weight of 14,900 whereas β -subunit contains 145 residues with a total molecular weight of 23,000. The carboxy-terminus of β -hCG contain 30 amino acids which are not present in the β -subunit of hLH. This extra sequence of β -hCG is potentially of great significance in providing an immunological basis for distinction between hCG and LH.

TABLE 1

Number of amino acid residues per molecule of hCG subunits
(Morgan et al, 1975)

Amino acids	α -hCG	β -hCG
Alanine	5	8
Arginine	3	12
Aspartic acid/Asparagine	6	11
Cystine (half)	10	12
Glutamic acid/Glutamine	9	9
Glycine	4	8
Histidine	3	1
Isoleucine	4	12
Lysine	6	4
Methionine	3	1
Phenylalanine	4	2
Proline	7	22
Serine	8	13
Threonine	8	10
Tyrosine	4	3
Valine	7	12

TABLE II

Carbohydrate composition of hCG and its subunits (gm/100 gm)

	a	b	Native hCG		α-hCG		β-hCG	
			c	d	e	f	e	f
Galactose	4.9	3.9	5.3	5.0	5.7	3.2	6.3	6.3
Mannose	4.9	2.9	5.3	4.8	5.3	6.6	3.0	3.0
Fucose	1.2	0.6	0.6	0.6	Trace	ND	Trace	Trace
N-acetylglucosamine	7.0	7.3	8.9	8.8	7.9	9.0	7.0	7.0
N-acetylgalactosamine	1.7	1.8	2.1	1.8	2.6	ND	3.4	2.5
Sialic acid	8.5	8.2	9.0	8.4	9.1	7.3	8.3	7.3
a =	Got et al (1960)							
b =	Goverde et al (1968)							
c =	Bahl (1969a)							
d =	Mori (1970)							
e =	Canfield et al (1976)							
f =	Morgan et al (1974)							
ND =	not detected							

Ala — Pro — Asp — Val — Gln — Asp — Cys — Pro — Gln —
 Cys₁₀ — Thr — Leu — Gln — Glu — Asp — Pro — Phe — Phe —
 Ser — Gln₂₀ — Pro — Gly — Ala — Pro — Ile — Leu — Gln —
 Cys — Met — Gly₃₀ — Cys — Cys — Phe — Ser — Arg — Ala —
 Tyr — Pro — Thr — Pro₄₀ — Leu — Arg — Ser_{CHO} — Lys — Lys —
 Thr — Met — Leu — Val — Gln₅₀ — Lys — Asn_{CHO} — Val — Thr —
 Ser — Glu — Ser — Thr — Cys — Cys₆₀ — Val — Ala — Lys —
 Ser — Tyr — Asn — Arg — Val — Thr_{CHO} — Val₇₀ — Met — Gly —
 Gly — Phe — Lys — Val — Glu — Asn_{CHO} — His — Thr₈₀ — Ala —
 Cys — His — Cys — Ser — Thr — Cys — Tyr — Tyr — His₉₀ —
 Lys — Ser

Fig.1(a) The amino acid sequence of alpha-subunit of hCG **according** to Morgan et al (1975).

The sites of attachment of oligosaccharide chains (CHO) are indicated.

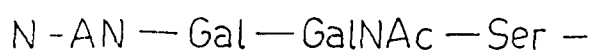
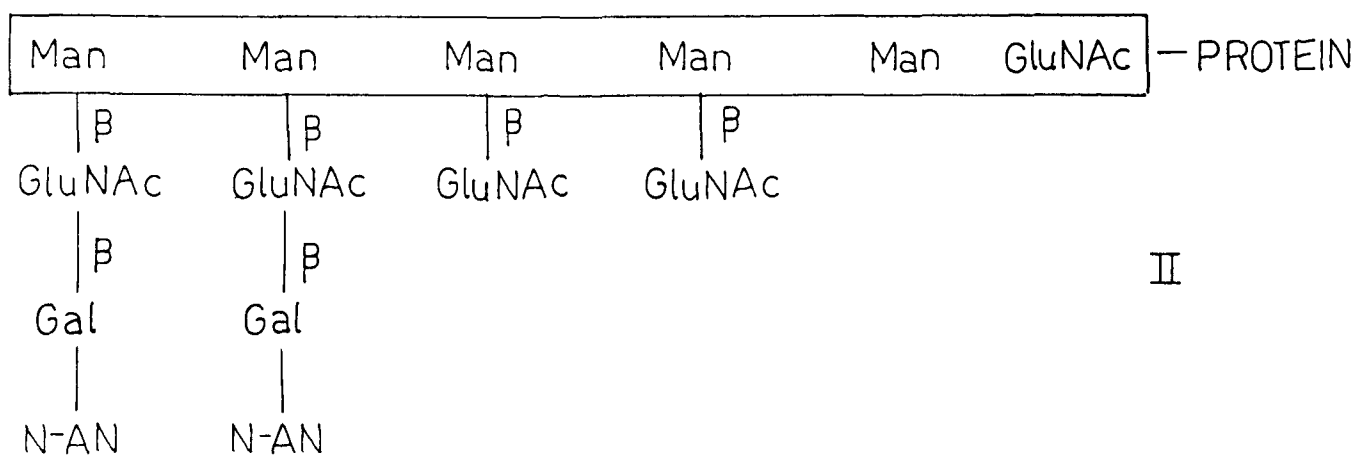
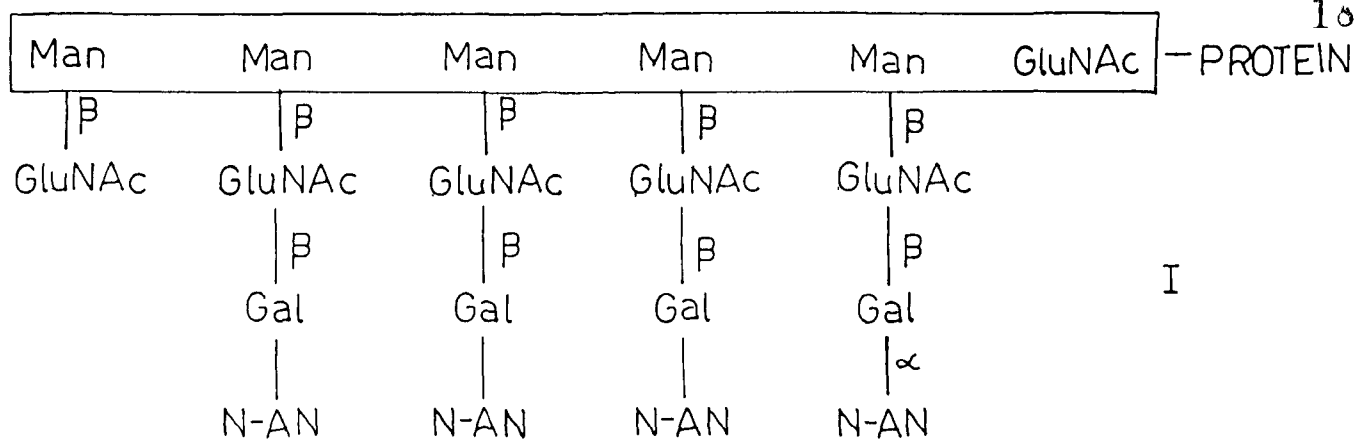
Each subunit is highly cross-linked by disulfide bonds, of which there are five in α -subunit and six in β -subunit. The pairing of the half cystine residues has not yet been established with certainty.

(iii) Carbohydrate composition

HCG contain approximately 30% carbohydrate, which is made up of six different monosaccharide units: sialic acid, L-fucose, D-galactose, D-mannose, and N-acetylglucosamine and N-acetylgalactosamine (Table 2). HCG is unique among glycoprotein hormones in its high content of sialic acid and its consequent long circulatory half life.

The first tentative proposal for the arrangement of monosaccharides in hCG has been presented by Bahl (1969a). He showed evidence for the presence in hCG of both types of carbohydrate-protein linkages commonly found in glycoproteins, namely the N-glycosidic linkage between N-acetylglucosamine and an asparagine residue and the O-glycosidic linkage involving N-acetylgalactosamine and a serine residue. He proposed two structures for large chains (structures I and II Fig.2) and another structure for the short serine-linked chain (structure III Fig.2). In a later communication, Bahl (1971) assigned structure I and structure II to α - and β -subunits respectively.

The structure of the carbohydrate chains of α -subunit has been studied by Kennedy and Chaplin (1976). In contrast to studies of Bahl (1969a) which used the carboxamidomethylated



III

Fig.2. Structures for oligosaccharide chains of hCG proposed by Bahl (1969). Man = Mannose; Gal = galactose; GluNAc = N-acetylglucosamine; GalNAc = N-acetylgalactosamine and N-AN = N-acetylneuraminic acid (sialic acid)

derivative of hCG, these workers used modified α -subunit. Proteolysis with trypsin gave rise to various peptides and glycopeptides which were identified. By subjecting the two glycopeptides to component analysis, it was found that these represented the two carbohydrate chains of the parent molecule. By sequential removal with specific glycosidases of monosaccharide units from these two glycopeptides. It was demonstrated that:

- (i) galactose, mannose, glucosamine and sialic acid possess the D-configuration
- (ii) glucosamine units were N-acetylated
- (iii) in the oligosaccharide chain, sialic acid units were farthest from the peptide backbone, followed by D-galactose units

They also demonstrated that the D-galactose, D-mannose and N-acetylglucosamine units were present in the pyranose form, that the D-galactopyranose units were linked in the 1 and 6 position; and that the D-mannopyranose units were present in several forms i.e. one in a terminal non-reducing position, one as a 1,2-linked residue, some as 1,6-linked residues and some as 1,2,6-linked branch points. The N-acetylglucosamine units were 1,6-linked.

(iv) Physico-chemical properties

Molecular weight determinations of hCG have given a wide range of values. Van Hell et al, (1968) derived a value of about

62,000 by sedimentation equilibrium. Bahl (1969b) reported a value of approximately 59,000 by gel filtration and 47,000 by sedimentation equilibrium. The higher value obtained by gel-filtration is consistent with the results obtained with other glycoproteins (Whitaker, 1963). The calculated molecular weight of hCG, from the composition of subunits is 38,000 for β -subunit and 15,000 for the α -subunit (Bellisario et al, 1973).

(v) Biosynthesis of hCG

The hormone production by placenta has been confirmed only in humans and other primates. Tullner (1974) has reviewed data available for primates. The site of biosynthesis of hCG is now accepted to be predominantly in the syncytiotrophoblast of the placenta (Midgley and Pierce, 1962; Mason et al, 1969; Okudaira et al, 1971). The mechanisms controlling biosynthesis, storage and release of hCG are not known.

2. Biological Polymorphism

Studies on the molecular forms of hCG in plasma, urine and placental extracts (Franchimont and Reuter, 1972; Vaitukaitis, 1974; Vaitukaitis et al, 1976) indicated the presence in all three biological materials of native hCG and of the α -subunit which was immunologically similar to α -subunit of hCG. In addition to native hCG, placental extracts in the second trimester of pregnancy, contained a larger molecular form of the α -subunit. Little β -subunit was detected in the plasma and in

placental extracts but a low molecular weight substance was found in urine which reacted in a radioimmunoassay for β -hCG.

A large immunological species of hCG was also reported by Tojo et al (1977) in extracts of chorionic tissue cultivated in vitro and in culture media. This large species of hCG, also eluted in the void volume during Sephadex G-100 chromatography, could not be distinguished immunologically from purified hCG but had low biological activity in the radioreceptor assay. Since the large immunological species was the predominant form in chorionic tissue cultivated for a short period, and the authentic hCG was the predominant form in the media, the authors suggested that the large hCG, possibly a prohormone, synthesised initially in the placenta, may be converted to the active form before secretion into the media.

The question of a hCG prohormone is still unsolved, but the finding (Landefeld, 1976) of a mRNA in placental tissue which in an in vitro system gave rise to a peptide of MW 16,000 which was very similar to α -hCG, argues against the concept of a single chain prohormone for hCG.

3. Mechanism of Action

It is generally thought that the binding of hormones to membrane receptors in the initial event leading to activation of adenylate cyclase and subsequent production of a specific target tissue response. Human LH and hCG appear to bind to the

same receptors in a variety of species. The binding is principally localised to the plasma membranes, as evidenced by subcellular fractionation techniques (Rajaniemi et al, 1974; Rao, 1973) and electron microscopic radiography (Han et al, 1974). The binding of hCG and hLH to common receptors in rat testis and ovary has been studied extensively (Catt and Dufau, 1973; Catt et al, 1974). Similar binding sites have been demonstrated in porcine granulosa cells in vitro and in vivo (Channing and Kammerman, 1974). In vitro experiments with particulate fractions from luteinised rat ovaries (Lee and Ryan, 1971; 1972) indicated that the binding of labelled hCG was specific, saturable and dependent on time temperature and ionic strength. Binding is of high affinity with dissociation constant of 10^{-11} M.

Whereas during in vitro studies there is a high specificity of binding of hCG to gonads, but liver and kidney have been found to incorporate significant amounts of hCG label (Ashitaka and Koide, 1974; Rao and Saxena, 1973).

A number of studies have been reported on the isolation and characterization of hCG receptors from different target organs (Lee and Ryan, 1974; Haour and Saxena, 1974). The hormone appears to stimulate the synthesis of its own receptor. Pandian et al (1975) showed that hCG stimulate the incorporation of [14 C] N-acetyl-D-glucosamine in rat ovary in vitro and that an appreciable amount of radioactivity was incorporated in the cell surface receptor for hCG. In addition to detergent-solubilized gonadotropin receptors, soluble gonadotropin-binding

factors have been isolated from rat testis (Bhalla et al, 1976). The dissociation constant of ^{125}I -hCG binding to soluble factors was similar to that derived for membrane-bound or detergent-solubilised receptors.

Evidence that hCG may regulate the number of its own receptors has been presented by Sharpe (1976). His studies with immature rat testis in vitro and in vivo suggested that the binding of hCG to specific gonadotropin receptors in the testis affected the subsequent availability of these receptors.

4. Concentration of hCG and Subunits in Placental Tissue

Bioassay estimates, of the concentration of hCG in first trimester placenta gave values in the range of 100-650 μg of wet tissue and average values were lower in the last trimester (Diczfalusy, 1953). Studies by Vaitukaitis (1974) showed that all the placental extracts studied contained hCG and α -hCG but the concentrations of the two glycoproteins varied qualitatively and quantitatively during progression of pregnancy. The absolute amount of hCG and α -hCG decreased after the first trimester but the relative quantity of α -hCG compared with intact hCG increased by more than 10 fold in the last two trimesters. An additional species of α -subunit was identified in the second trimester.

5. Distribution of hCG in Body Fluids

Although a correlation between individual maternal and foetal concentrations of hCG have been found, the concentration is

much higher in maternal blood than in foetal blood. The ratio of maternal-foetal concentration was found to be 344:1 (Geiger et al, 1971). Somewhat higher values for the maternal/foetal ratio have also been reported (Midgley et al, 1967; Crosignani et al, 1972). A much lower value was reported by Faïman et al (1968). Amniotic fluid concentrations also show a correlation with plasma concentrations and the evidence suggests that values are higher in early pregnancy than at term.

The concentration of hCG in extra-cellular fluid has not been reported. Concentration in cerebrospinal fluid (CSF) remains substantially lower than the plasma concentration even when plasma values have been at a high level for long periods of time. In patients with trophoblastic tumours who had no evidence of intracranial metastases, the plasma/CSF ratio was between 60/1 and 600/1 for a wide range of values, with a mean value in excess of 200/1 (Bagshawe et al, 1968). There was a significant time lag between changes in the plasma concentration and changes in the CSF concentration of hCG.

6. Renal and Metabolic Clearance in Man

Bioassay estimates of the rate of renal clearance of hCG have ranged from 0.18 to 1.19 ml/min (Gastineau et al, 1949). Values obtained during the first, second and third trimesters of pregnancy by radioimmunoassay were 0.94, 0.66 and 0.85 (Crosignani et al, 1971). One study found a mean clearance rate of 0.36 ml/min by bioassay but a value of 0.70 ml/min by

immunoassay as a result of differences between bioassay and immunoassay estimates of plasma values (Wide et al, 1968). Clearance values estimated by radioimmunoassay on more than 100 patients with trophoblastic tumours have been 0.5 to 1.8 ml/min for plasma concentrations. The plasma and urine concentration of hCG tend to be similar when the daily urine volume is about 1-5 liters simply because the clearance rate approximates 1 ml per min.

Early studies indicated that about 20 percent of an intravenously administered preparation of hCG could be recovered from the urine (Friedman and Weinstein, 1937). Later studies indicated that similar figure was obtained when hCG administered i.v. or i.m. to amenorrhoeic women was expected in the urine during the succeeding 5 to 6 days (Wide et al, 1968). Following i.v. injection, a metabolic clearance rate of 3.38 for men and 3.86 for women was found with initial fast component half-lives of 5.1 hrs and 5.6 hrs and slow component half-lives of 23.6 hrs and 23.9 hrs in men and women respectively (Rizkallah et al, 1969).

7. Distribution and Clearance on Experimental Animals

When hCG is administered to another species, the pattern of distribution and excretion may be influenced by various factors which do not operate in man. About 75 percent of hCG administered i.v. to rabbits was recorded from the urine over a 4 to 24 days period using an unspecified immunoassay method (Hisley and Fesche, 1966).

Administering ^3H -labelled hCG and α -hCG and β -hCG subunits to immature female rats, Braunstein et al (1972) found biphasic plasma disappearance curves with fast and slow components having $t_{1/2}$ times of 141, 6.2 and 11.1 min for the fast phase, and 725, 58 and 81 min for the slow phases respectively.

8. HCG in Neoplastic Diseases

(1) Trophoblastic and other tumours

Classical hydatidiform mole is an abnormal conceptus in which there is generally an excess of trophoblast associated with hydropic villi and little, if any, foetal tissue proper. Partial degrees of mole can also occur. The trophoblast of hydatidiform mole or that of normal non-molar pregnancy may give rise to the malignant tumour choriocarcinoma. Choriocarcinoma also arises in teratomas of the testis and ovary. All these lesions are regularly associated with the production of hCG (Masune et al, 1981; Nishimura, et al, 1981; Amr et al, 1983). The detection of hCG in the body fluids of anyone who is not pregnant and who has not recently had a hydatidiform mole is indicative of a malignant tumour. In the case of patients who have recently had a mole, the persistence of hCG in body fluids for several months indicates the presence of either invasive mole or choriocarcinoma. Metastatic deposits in the lung of a 27 year old woman associated with invasive trophoblastic hydatidiform mole were detected only with the aid of blood pool subtraction

technique using a polyclonal and monoclonal antibody specific to hCG (Morrison et al, 1984). Cancer radioimmunodetection with antibodies to hCG and to alpha fetoprotein appear to be a useful procedure for the evaluation of patients with testicular cancer and can reveal sites of tumour not detected by other methods (Javadpour et al, 1980; Begent et al, 1980; Goldenberg et al, 1981; Wu et al, 1983).

(ii) Hydatidiform mole

Zondek (1929) recognised the production of hCG by hydatidiform moles and further notable contributions were made by Hamberger (1944) and by Delfs (1959). It is widely held that hCG values in plasma and urine are greatly increased when mole is in situ in the uterus. This appears to be true in only half of all cases (Campbell et al, 1970). Values within the normal pregnancy range are found and in some instances, may be below the normal values for that stage of pregnancy. In general the concentrations of α -hCG and β -hCG are much lower than the corresponding concentrations of intact hCG. No difference in the three groups of patients with complete hydatidiform moles, partial hydatidiform mole and non-molar abortions (Smith et al, 1984). The risk of choriocarcinoma after mole is about 1000 times higher than following normal term pregnancy and the risk is largely, although not completely, confirmed to the first two years. In majority of patients, the trophoblast of hydatidiform mole dies out spontaneously but it commonly takes much longer for this to

to be complete than after normal pregnancy, and molar trophoblast may persist for many months as invasive mole (Bagshawe, 1969). An apparent degree of correlation was observed with tumour activity in that case with widespread metastases due to a colonic carcinoma exhibited the highest hCG levels while, in one patient, the level of hCG decreased progressively according to therapeutic response (Ayala et al, 1983). A high frequency of immunoactive hCG was found in carcinomas of the cervix, breast, gonads and digestive system and in malanomas (Ito and Tahara, 1983; Jacobsen and Jacobsen, 1983; Bychkov et al, 1983; Morinaga et al, 1983).

(iii) Ectopic production of hCG by non-trophoblastic tumors

Gonadotropin production by patients with carcinoma of the lung was described by Fusco and Rosen (1965). Braustein et al (1973) studied detectable levels of hCG in patients with non-testicular tumors, carcinoma of the stomach, pancrease or breast, multiple myloma and melanoma were those most likely to give positive response (Goldstein et al, 1974). In patients with islet cell tumors, hCG and its subunit were found only in those with islet cell carcinoma (Kahn et al, 1977). Hormone production was also found in patients with hepatocellular carcinoma (Braustein et al, 1973)..

9. Immunological Properties of hCG

The intact molecule, free alpha and beta subunits and natural fragments of hCG are all immunogenic and antigenic

(Musch et al, 1981; Nieschlag et al, 1982; Stuart et al, 1983). Moreover knowledge of the amino acid sequences has made it possible to synthesize carbohydrate free replicas of beta-subunit fragments which are both immunogenic and antigenic (Thanawala et al, 1980; Chang et al, 1981; Caraux et al, 1985; Bellet et al, 1987). The properties have been exploited to produce reagents and assays used to study the structure function determinants of immunologic and biological properties of the hormone (Kofler et al, 1982; Furuhashi et al, 1983; Searle et al, 1984).

Demonstration of partial cross-reactivity of hLH in the assay system based upon this particular suggested that it might be possible to produce an antiserum which would be less cross-reactive and therefore capable of distinguishing hCG from hLH, hFSH and hTSH in mixtures of these hormones. Such an antiserum was identified and a satisfactory assay developed for clinical use (Thau et al, 1983).

The frequency with which antisera specific for hCG and β -hCG can be produced is enhanced by immunizing rabbits with a conjugate of an appropriate carrier with either natural or synthetic peptide consisting of 23 of the 30 carboxy-terminal amino-acids unique to the β -subunit of hCG (Nash et al, 1980; Stevens et al, 1981; Birken et al, 1982; Covey and Laurance, 1984). Virtually all such antisera distinguish the β -subunit of hCG from all other human glycoprotein hormones.

Antigenic potencies of synthetic peptides, produced by successively adding appropriate amino acids to the carboxy-terminal amino acid, have been compared (Chen et al, 1976). In these assay, addition of amino acid beyond its carboxy-terminal residues did not result in any further enhancement of antigenic potency of synthetic peptides which were equipotent with both native and S-carboxymethylated or S-amidomethylated β -subunits of hCG (Ross, 1977).

10. Monoclonal Antibodies in Study of Structure Function Relationship

The study of protein structure at all levels proceeds via a series of well known technique worked out over the last 20-30 years (Hirs, 1967). The relationship of protein function to protein structure is a rather specialized science as is the immunochemical analysis of surface structure on proteins. The recent technique of monoclonal antibody production promises to have an enormous impact on these three areas of scientific research after the initial application being mainly to all surface markers (William, 1977; Parham et al, 1982). This is mainly because the monoclonal antibodies bind to single specific points on the protein antigen and they are available in large quantities and invariant quality. These properties combine to make the study to protein structure with these reagents much easier.

Studies on immunochemical analysis of proteins by polyclonal antibodies have been reviewed (Cinader, 1967; Arnon, 1973; Atassi, 1977). A chemical technique to myoglobin elucidate its

complete immunochemical structure using polyclonal antisera has been published by Atassi (1975).

There have been a number of reports on the production of monoclonal antibodies to hCG (Kofler et al, 1982; Moyle et al, 1982; Chow et al, 1986). Monoclonal antibodies to hCG can be used to quantitate hCG in a 2-site sandwich radioimmunoassay (Mizuchi et al, 1984). Wada et al (1982) described sandwich enzyme immunoassay of hCG with solid phase monoclonal antibody for the α -subunit and enzyme coupled monoclonal antibody, specific for the β -subunit. Recently capillary tube enzyme immunoassay and luminescent immunoassay were used for quantitation of human chorionic gonadotropin (Longeon et al, 1986; Nagainis, et al, 1986).

Totally specific anti-hCG antibodies are directed towards an epitope containing amino acids on both α -and β -subunit but spatially arranged so that such antibodies cannot recognize these amino acids in the separate subunit molecule (Stuart et al, 1983). The production of monoclonal antibodies directed toward hCG and its subunit after immunization with hCG (Gupta and Talwar, 1980; Stahl et al, 1980) and its β -subunit (Ehrlich and Canfield, 1982; Khazaeli et al, 1981) have also been reported. Monoclonal antibodies against each subunit have mapped the relative binding sites of several monoclonal antibodies that react with intact hCG using a solid phase assay (Moyle et al, 1982, 1983).

One of the essential advantages of MAb compared to conventional polyclonal antisera is certainly their monospecificity

for a particular antigenic determinant on a complex molecule. Nevertheless, it is obvious that in situation where the recognized determinant is present in the identical or conformationally similar form on different molecule, a 'true' immunological cross reactivity will be revealed. However, in determining the quality and possible application of an antibody preparation the antibody affinity must be atleast equally as important as the specificity. Since the affinity is directly related to the sensitivity of the test system in which the antibody will be used. This holds true for most hormone determinations using immunological techniques where apart from high specificity, high sensitivity is required due to the low concentrations of the compounds investigated (Berger et al, 1984).

11. Immunological Properties of β -Subunit of hCG

The α -subunit of the hormone is chemically identical to the α -subunits of such hormones as LH, hFSH and hTSH which are circulating continuously in the body. Isoimmunization with hCG results in the production of antibodies which cross react with all these hormones, leading to severe endocrinological disturbances (Stevens, 1983; Stevens and Crystle, 1973) and the danger exists of possible immunopathological changes in susceptible organs, particularly the pitutary. The β -subunit of these hormones are different and confer biological activity (Canfield et al, 1971; Pierce et al, 1971a). A fertility regulation vaccine based on the β -subunit of hCG would therefore seem a more promising proposition. Several investigator have used this

approach in a variety of animal species including baboons, marmosets, Rhesus monkey, sheep and rabbits (Stevens, 1975; Hearn et al, 1975; Stevens 1976; Talwar et al, 1976a) and in women (Talwar et al, 1976b; Nash et al, 1980; Shahani et al, 1982). However, although immunization with β -hCG results in the production of a good response in terms of amount and affinity of antibodies against hCG, cross-reactivity with hLH is still present (Thanwala et al, 1978).

The antigen used in the vaccine was limited to the β -subunit of hCG to minimize the cross-reactions of antibodies with other glycoprotein hormones. It was hoped that linking the β -subunit to a foreign protein would render it antigenic to the human being. Tests of a β -hCG-TT vaccine in human showed to stimulate antibodies against hCG but the titers were relatively low and transient in some of the test subject (Talwar et al, 1976b; Nash et al, 1980; Shahani et al, 1982).

Initial trials of such a vaccine in women (Nash et al, 1980) showed that antibodies capable of neutralizing the biological activity of hCG were produced but the titers varied considerably among individuals and in some women were very low. It was therefore deemed necessary to increase the antigenic response by adjuvants. The efficacy of alternative protein and carbohydrate carriers and of the muramyl dipeptide family of adjuvants in effecting increased antibody response in an animal test system has been reported (Tsong et al, 1985; Nash et al, 1985). The effectiveness of adjuvants in increasing antibody

response in rabbits to vaccination with the β -subunit of hCG linked to tetanus toxoid (Nash et al, 1985; Chang et al, 1985) have been reported. Several adjuvants including some analogs of muramyl dipeptide and a streptococcal preparation OK 432 increased antibody titer to 3-10 folds (Laurence and Covey, 1984; Change et al, 1986). But the vaccine consisting of the β -subunit of hCG linked to TT and adsorbed on $Al(OH)_3$ suggested that the magnitude and duration of the antibody response were not adequate to prevent pregnancy in some women (Nash et al, 1980; Shahani et al, 1982).

Corynebacterium parvum was compared with Freund's complete adjuvants for potentiation of the rabbit immune response to β -subunit linked to tetanus toxoid. With each adjuvant, antibodies to hCG were detected using passive hemagglutination. Higher antibody titer were produced by FCA treated animals (Covey and Laurence, 1984).

12. C-Terminal Peptide of β -Subunit of hCG

Antisera raised against the entire β -subunit of hCG usually cross-react to some extent with human LH. While this degree of cross-reaction in serum assay can be relatively minor, human LH becomes a greater source of error when urine specimens are concentrated for measurement of very low levels of hCG secretion. To circumvent this problem peptides from the unique β -COOH-terminal region can be employed as immunogen to elicit antisera of greater specificity for hCG. The use of β -carboxy terminal

peptide as immunogen with the full complement of carbohydrate that have given rise to antisera of improved sensitivity for hCG detection has been reported (Birken et al, 1982).

The specific biological activity of highly purified hCG was determined by a ventral prostate weight bioassay before and after incubation with an antiserum to a COOH-terminal peptide unique to the β -subunit of hCG (Louvét et al, 1974). Although the antibody was shown to bind hCG, the antibody hormone complex retained the biological activity of the native hormone. The author have reported that immunization with the COOH-terminal polypeptide region of the β -subunit does not necessarily stimulate production of neutralizing antibodies (Louvét et al, 1974). While the non-cross reactive monoclonal antibodies to hCG were prepared after immunization with a conjugate of synthetic peptide to diphtheria toxoid (Caraux et al, 1985).

But in a study, a peptide fragment analogous to the amino acid sequence of the carboxyl terminal 45 residues (101-145) of the β -hCG with α -aminobutyric acid substituting for cysteine at position 110 was synthesized. Antisera raised against this conjugate was found to bind with hCG but did not cross-react with hLH in radioimmunoassay (Chang et al, 1981). Most importantly, this antisera effectively neutralized the biological activity of hCG as determined by rat uterine weight assay. Similarly lack of cross-reactivity of the antibodies against a synthetic 37 amino acid C-terminal peptide of β -hCG conjugated to tetanus toxoid as carrier with hCG was determined by direct

binding in RIA and by immunofluorescence assay on adult human pituitary secretion (Thanwala et al, 1980).

13. Interaction of Furocoumarins with Proteins and Enzymes

The furocoumarins, also referred as psoralens, belong to a group of heterocyclic compounds formed by the condensation of coumarin and furan ring. Many natural and synthetic furocoumarins have been made in the laboratory (Scott et al, 1976). Of these furocoumarins, psoralen, 8-methoxypsoralen and 4,5,8-trimethylpsoralen are used clinically in the treatment of vitiligo and have been extensively investigated in terms of their therapeutic usefulness, photosensitivity and melanogenic properties (Fowlks, 1959; Kaufman, 1960; Scott et al, 1976).

The beneficial effects of the combination of psoralen plus 365 nm light (PUVA) in the treatment of skin diseases such as psoriasis control excess cell division in the skin by virtue of its ability to damage DNA. Such lesions in DNA thus lead to inhibition of DNA synthesis, erythema production and increased skin pigmentation as well as mutation and killing of bacteria, chromosome aberrations, inactivation of viruses and inhibition of tumour transmitting capacity of certain tumour cells. In addition to damage to DNA, PUVA treatment may also cause damage to proteins. Lens proteins are particularly susceptible to this type of damage since psoralen-protein photoadducts may be permanently lodged within the lens (Lerman and Brokman, 1978; Lerman et al, 1980). Apart from the photobiological consequences

of PUVA treatment, the photolabelling of biomolecules with psoralens provides a relatively new tool for probing biopolymer structure (Hallick et al, 1978; Nass, 1979; Shen and Hearst, 1979; Song and Ou, 1980).

As with DNA, psoralen form complex with protein. Several studies have concerned the interaction of furocoumarins with proteins (Talib, 1975; Veronese et al, 1978; 1979; 1981), so as to understand their role in the biological activity of furocoumarins. The strong non-covalent binding in the dark between furocoumarin and albumin suggests a possible influence of plasma proteins in the pharmacokinetic behaviour of these drugs (Veronese et al, 1978).

The association constants of psoralen with human serum albumin are similar to those with DNA (Artuc et al, 1979; Veronese et al, 1979). The ability of 8-methoxypsoralen (8-MOP) to bind with human serum albumin has been investigated in vitro through equilibrium dialysis and fluorescence quenching experiments (Veronese et al, 1978). 8-MOP also form a covalent conjugate with bovine serum albumin possibly with tyrosin residues by UVA irradiation under air. There is apparently no observable reaction between 8-MOP and tryptophan when the mixture in solution is irradiated in the absence of oxygen (Lerman et al, 1980). In the presence of oxygen, however, the loss of aromatic type hydrogen atoms at the 3', 4' and 4', 5' position of 8-MOP and at the indole C₂ position of tryptophan

has been shown (Megaw et al, 1980; Megaw and Lerman, 1980). No explanation was offered to account for the effect of oxygen, but a mechanism similar to that given for the photobinding of 8-MOP to proteins could be expected to prevail (Yoshikawa et al, 1979). In the latter work, it was demonstrated that the product formed in the reaction between 8-MOP and singlet oxygen readily add in the dark to bovine serum albumin to form a covalent conjugate. In the same study, it was also observed that some other form of the conjugate was produced via an oxygen-independent pathway and therefore, direct photoaddition of 8-MOP triplet state of the protein was proposed. In some studies, it is indicated that following UV-A irradiation, proteins may be modified since enzymes may be inactivated by the photodynamic action of 8-MOP probably via singlet oxygen production (Poppe and Grossweiner, 1975; Veronese et al, 1979) and covalent photoaddition of the furocoumarin to protein was observed (Yoshikawa et al, 1979; Lerman et al, 1980).

In the present study, commercial sample of hCG has been purified to homogeneity by column chromatography. The effect of UV irradiation on hCG has been investigated by spectroscopic methods. Tryptic digestibility, urea denaturation, lysine modification of hCG and its modified form along with immunological characterization of its induced antibodies have also been investigated. Dark interaction of psoralen with hCG and its α and β subunits was carried out by fluorescence quenching technique. The validity of this technique is supported by the

fact that interaction studies using fluorescence quenching and equilibrium dialysis technique yields essentially the same results (Romeu et al, 1976). The specificity and cross-reactivity of their induced antibodies have been investigated by ELISA. Different peptides of β -subunit were isolated by chymotrypsin digestion. These were separated and purified by column chromatography and preliminary studies identified the reactive fragment(s).

EXPERIMENTAL

(A) MATERIALS

Proteins

Human chorionic gonadotropin (hCG), bovine serum albumin, α -chymotrypsin and trypsin were obtained from Sigma Chemical Company, U.S.A.

Chemicals for Polyacrylamide Gel Electrophoresis

Acrylamide and Coomassie brilliant blue R were purchased from Sigma Chemical Company, U.S.A. NNN'N'-tetramethylethylenediamine, sodium dodecyl sulfate and ammonium persulfate were the product of B.D.H. England. Bisacrylamide was from Reanal, Hungary and β -mercaptoethanol was purchased from Calbiochem, U.S.A.

Chemicals for Immunization

Chromatographically pure psoralen was a gift from Central Drug Research Institute, Lucknow, and Freund's complete adjuvant was obtained from Difco Chemical Company, U.S.A.

Chemicals used in Enzyme Linked Immunosorbent Assay

Goat anti-rabbit IgG alkaline phosphatase conjugate was purchased from Sigma Chemical Company, U.S.A. p-nitrophenyl phosphate was from C.S.I.R. Centre for Biochemicals, Delhi. Diethanolamine was from B.D.H. India. Tween-20 was from Koch-light Laboratories, England, and sodium azide was obtained from

Polskie, Poland. Polystyrene flat bottom plates having 96 wells were purchased from Cooke Microtiter Company, U.S.A.

Miscellaneous Chemicals

Agarose and 2,4,6-trinitrobenzene sulfonic acid were purchased from Sigma Chemical Company, U.S.A. Sephadex G-100, G-50, G-25, Blue dextran-2000, Sepharose 4B were obtained from Pharmacia Fine Chemicals, Sweden. Ninhydrin was from B.D.H. England. DEAE-cellulose was obtained from Whatman, England and cynogen bromide was from Sisco Research Laboratories, Bombay. All other chemicals used were of highest analytical grade.

Irradiation Source

Short wavelength ultraviolet lamp, Spectroline (Model R-51, Black Light Eastern Inc, U.S.A.) having maximum emission at 254 nm and long wavelength ultraviolet lamp, Spectroline (Model B-100, Black Light Eastern Inc, U.S.A.) having maximum emission at 365 nm, were used as the source of radiant energy.

Light Absorption Measurements

Spectral properties of proteins in UV and visible region were studied using Beckman DU-6 spectrophotometer with quartz cuvette of 1.0 cm path length. The fluorescence studies were conducted on Aminco-Bowman spectrophotofluorometer equipped with Xenon lamp as excitation source.

pH measurements

The pH was measured on an Elico pH meter model LI-10T (Electronics, Corporation, Hyderabad, India) with Elico glass and saturated calomal electrodes.

(B) METHODS

1. Purification of Human Chorionic Gonadotropin

The commercial samples of human chorionic gonadotropin obtained from Sigma Chemical Company, U.S.A. showed heterogeneity in polyacrylamide gel electrophoresis. The hormone was purified by gel filtration.

2. Gel Filtration Chromatography

Preparative gel filtration chromatography was performed on Sephadex G-100 column (1.8 cm x 66.0 cm) equilibrated with 0.05M phosphate buffer pH 7.5 containing 0.02 percent sodium azide. Commercial human chorionic gonadotropin (20-25 mg/ml) was applied to the column and eluted with equilibrating buffer at a flow rate of 20 ml/hr in 3.0 ml fractions. The fractions were monitored from protein at a wavelength of 280 nm. The peak fractions were pooled, concentrated and dialyzed against the same buffer.

3. Polyacrylamide Gel Electrophoresis

The purity of human chorionic gonadotropin was checked by native polyacrylamide disc gel electrophoresis (Laemmli, 1970).

- (a) Buffers and other reagents used were as follows:
- (i) Acrylamide solution (30% acrylamide and 0.8% bisacrylamide)
 - (ii) Lower Tris (0.37M Tris pH 8.8 adjusted with 6 N HCl)
 - (iii) Upper Tris (0.062M Tris, pH 6.8, adjusted with 6 N HCl)
 - (iv) Reservoir buffer (Tris-glycine, pH 8.3)
 - (v) Sample buffer (upper Tris buffer contains 1M sucrose)
 - (vi) Staining solution (0.25% Coomassie Brilliant Blue, 25% methanol and 10% acetic acid)
 - (vii) Destaining solution (20% methanol and 7% acetic acid).

The stock acrylamide solution was diluted to a 7.5 percent concentration for resolving gel (0.6 cm x 15 cm in Lower Tris) and to a 3 percent concentration for the stacking gel (0.6cm x 3cm in upper Tris). Protein samples were prepared in upper Tris containing 1M sucrose and 0.005 percent bromophenol blue as tracking dye. Usually 100 μ g of protein was electrophoresed. The electrophoresis was performed at 1mA/gel during the first hour of stacking and 2mA/gel until the tracking dye reached the bottom of the gel (5-6hr). The gels were removed and set into staining solution and thereafter destained in destaining solution.

Subunit composition of hCG was determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1960).

4. Separation of Subunits of Human Chorionic Gonadotropin

Separation of subunits was done by two different methods.

- (i) Subunits were separated using the method of Canfield et al, (1970). Purified hCG was incubated in 3.0 ml of 10M urea in 3mM

sodium borate/7mM sodium phosphate pH 8.0 for 24 hrs at room temperature. After incubation the sample was applied onto a column of DEAE-cellulose previously equilibrated with borate/phosphate buffer. Alpha subunit of hCG (α hCG) was not retained by the column and therefore eluted with equilibrating buffer. Whereas beta subunit (β hCG) was eluted with equilibrating buffer contains 0.1M NaCl and 5M urea.

(ii) An other method of separation of subunits was the method of Swaminathan and Bahl (1970). Briefly, the dissociation of hCG into two subunits was effected by 8M urea. A sample of purified hCG (11.9mg) was dissolved in 0.1 ml of 8M urea in 0.04M Tris-phosphate buffer pH 7.5 and incubated at 37°C for 60 min. The incubation mixture was diluted in 0.25 ml with the above buffer and transferred to a column of DEAE-cellulose (0.9cm x 30cm), pre-equilibrated with 0.04M Tris-phosphate buffer, pH 7.5. The column was developed with a salt gradient (0.0 to 0.2M NaCl in 0.04M Tris-phosphate buffer pH 7.5) and two protein peaks, designated as A and B, corresponding to α and β subunits were obtained (Fig.3). The peak fractions were pooled, concentrated and exhaustively dialyzed against buffer.

5. Ultraviolet Irradiation of hCG

The hormone in 0.05M phosphate buffer, pH 7.5 was irradiated with 254nm light. The solutions were irradiated in small beakers placed in a water cooled container (Asif et al, 1969). Irradiation was carried out at a constant temperature of 25°C \pm 1°C.

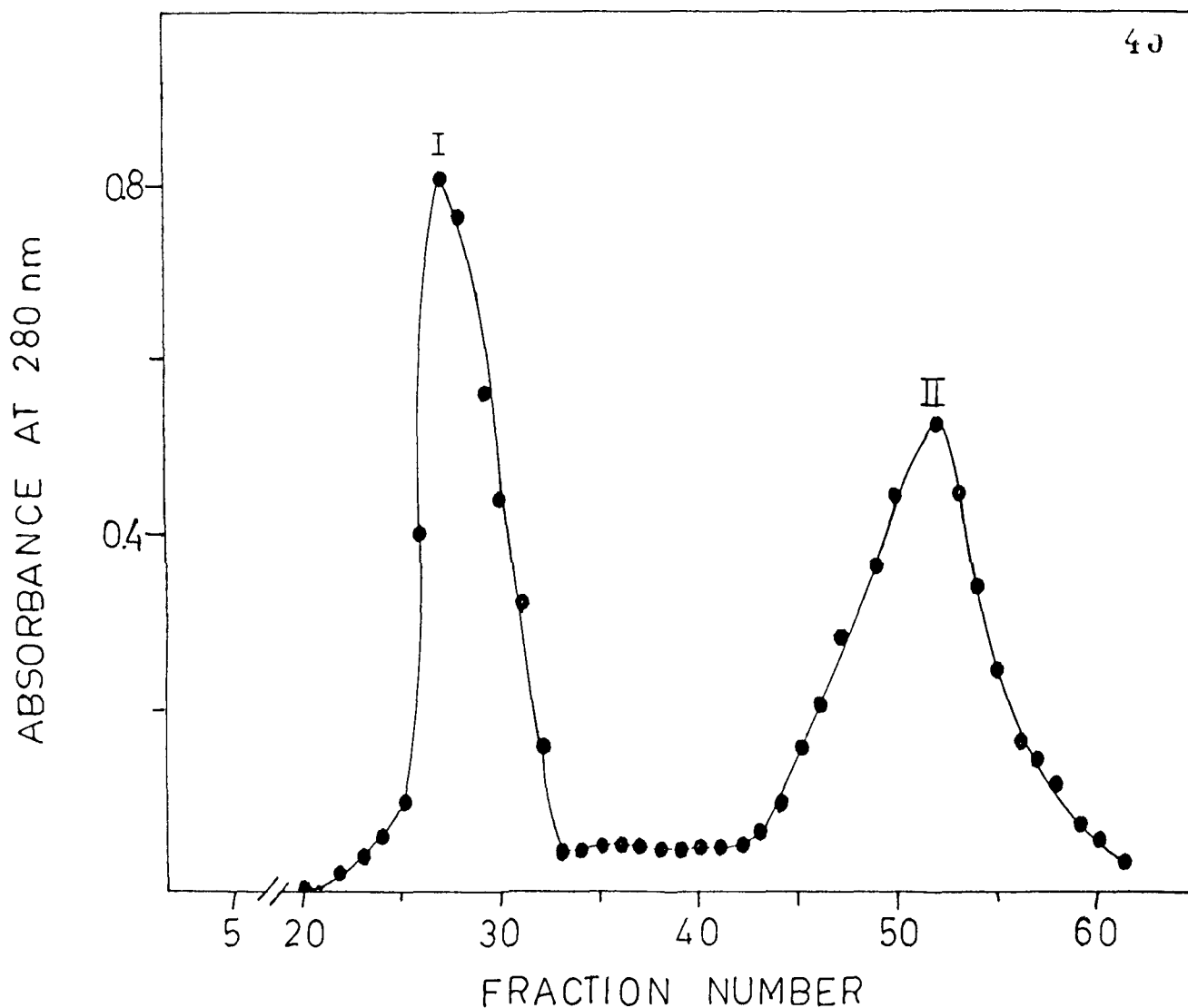


Fig.3. Separation of hCG subunits by DEAE cellulose chromatography.

The hormone was dissolved in 8M urea in Tris-phosphate buffer, pH 7.5 and incubated at 37°C for 1 hr. The sample was loaded onto a column (0.9x30cm) of DEAE cellulose and the bound material was eluted with a linear gradient of 0.0 - 0.2 M NaCl. Fraction size : 3.0 ml; Flow rate: 20 ml/hr.

6. UV Spectral Properties of hCG

The light absorption characteristics of native and irradiated hCG in UV region were studied on Beckman DU-6 spectrophotometer. The hormone samples (500 $\mu\text{g/ml}$) were filtered through Millipore filter, prior to the spectral studies.

7. Fluorescence Spectral Studies

The intrinsic fluorescence of native and irradiated hCG were monitored in 0.05M phosphate buffer, pH 7.5. The protein was excited at 280 nm for the measurement of fluorescence from tyrosine residues. All the fluorescence studies were performed on uncorrected spectra.

8. Chemical Modification of Amino Groups of hCG

The modification of amino groups of hCG with trinitrobenzene sulfonic acid (TNBS) was performed according to the method of Plapp et al (1971). Briefly the protein (2 mg/ml, native/irradiated) was dissolved in borate buffer (0.05M $\text{Na}_2\text{B}_4\text{O}_7$, pH 9.5) and a fresh solution of 0.02M TNBS in water was prepared. The reaction was initiated by the addition of 0.1 ml of TNBS solution to 1.0 ml of protein solution. The reaction kinetics was followed by monitoring the absorbance at 367 nm. The extent of trinitrophenylation of hCG was monitored directly in the reaction mixture by assuming a molar extinction coefficient of $1.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for the trinitrophenyl group.

9. Effect of UV Radiation on the Limited Proteolysis of hCG

The tryptic digestibility of native and irradiated hCG was carried out by the method described by Paik and Kim (1972) with some minor modifications. The method involves quantitation of free amino groups exposed as a result of proteolysis. For the determination of free amino groups, the procedure described by Moore and Stein (1948) was followed.

(i) Crystallization of ninhydrin

10 gm of commercial ninhydrin powder was dissolved in 150 ml of hot water. The hot solution was stirred with 10 gm of activated charcoal and filtered. The filtrate was left at 4°C for 72 hrs. The crystals were washed with ice cold water and dried over phosphorus pentoxide in a vacuum desiccator.

(ii) Ninhydrin reagent

800 mg of ninhydrin was dissolved in 20 ml of methyl cellosolve and mixed with 20 ml of 0.2M sodium citrate buffer, pH 5.0, followed by the addition of 0.68 ml of 9.8 percent stannous chloride suspension in citrate buffer. The reagent was prepared fresh before use.

(iii) Procedure

To 3.0 ml of hCG (400 µg/ml in 0.5M phosphate buffer, pH 7.2) preincubated for 10 minutes at 37°C was added equal volume of trypsin (400 µg/ml in 0.5M phosphate buffer, pH 7.2). The

contents were mixed and incubated at 37°C. At known intervals of time, 0.5 ml aliquots of the digestion mixture were quenched and mixed with 0.5 ml of 0.2M chilled citrate buffer, pH 5.0. To 1.0 ml of chilled reaction mixture was added 1.0 ml of ninhydrin reagent and the samples were heated on a boiling water bath for 15 minutes. The tubes were cooled in ice water and 5.0 ml of 50 percent ethanol was added to each tube. Absorbance of the sample was recorded at 570 nm. The number of free amino groups exposed after tryptic hydrolysis of hCG was computed from a standard curve drawn with isoleucine (0.7×10^{-7} to 3.8×10^{-7} moles).

10. Colorimetric Method for the Determination of Reducing Sugars in hCG

Determination of sugar contents in native/irradiated hCG was done by the method of Dubois et al (1956). 1.0 ml of hCG solution was mixed with 1.0 ml of freshly prepared 5% phenol. Five ml of conc. H_2SO_4 was added rapidly. The stream of acid should be against liquid surface rather than side of the test tube in order to obtain good mixing. Tubes were allowed to stand half-an-hour at room temperature. Color was stable for several hours. Absorbance of characteristic yellow-orange color was measured at 490 nm for hexoses. Blanks were prepared by substituting distilled water in lieu of hCG.

10. Immunization

All rabbits were bled before receiving initial injection in order to obtain a supply of preimmune rabbit serum. Rabbits

(9-12 months old, weighing 1.0 to 1.5 kg) were immunized with antigen suspended (native/irradiated hCG) in Freund's complete adjuvant by intramuscular injection in hind leg, weekly for three weeks. Each animals received 100 μ g of antigen per injection. Blood was collected at weekly intervals by heart puncture. Sera were separated and stored at -4°C ,

12. Immunological Studies

(1) Immunodiffusion (ID)

The precipitin reactions were performed by Ouchterlony double diffusion in 0.4% agarose.

5-6 ml of molten agarose (0.4% in PBS containing 0.1 percent sodium azide) was heated to boiling, filtered and poured into glass petri dishes (50mm x 15mm) and allowed to harden at room temperature. Wells having a diameter of 5 mm were cut in a typical seven well pattern. The petri dishes were stored at 4°C .

50 μ l of serially diluted antisera were placed in peripheral wells and antigen in the central well. The diffusion of antigen and antisera in agarose gel was allowed for 24-48 hrs in a moist chamber at room temperature.

(11) Counterimmunoelectrophoresis (CIE)

CIE was carried out in 0.6% agarose using microscopic slide (Kurata and Tan, 1976). Three ml of 0.6 percent agarose (in 0.025M barbital buffer, pH 8.4 containing 0.1 percent sodium

azide) was pipetted and spread uniformly onto a clean microscopic slide (25mm x 70mm) and allowed to solidify at room temperature. The slides were stored in a moist chamber at 4°C and used within three days.

Antigen was placed in cathodal wells and antisera in anodal wells (20 µl in each well). Electrophoresis was performed in 0.05M barbital buffer, pH 8.4 with a current of 3-4 mA per slide for 30-50 minutes. Non-specific precipitin lines were abolished by washing the slides with 5 percent sodium citrate solution. The slides were examined visually for precipitin lines and the results recorded.

13. Enzyme Linked-Immunosorbent Assay on Polystyrene Plates

(a) Buffers and other reagents

(i) Bicarbonate buffer (coating buffer)

0.015 M sodium carbonate
0.035 M sodium bicarbonate
0.02 percent sodium azide
pH adjusted to 9.6

(ii) Saline Tween-20 (ST-20)

0.15 M sodium chloride
0.05 percent Tween-20
pH adjusted to 7.0

(iii) Diethanolamine buffer (DEA buffer)

1M diethanolamine

0.1 mg/ml magnesium chloride

0.02 percent sodium azide

pH adjusted to 9.8 with HCl

(iv) Substrate (PNP-P)

5×10^{-3} M p-nitrophenyl phosphate in diethanolamine buffer.

(b) Procedure

Technique of Voller et al (1976) with some modifications was used for the detection of antibodies. Hormone in coating buffer (1 μ g/0.25 ml/well) was added to the wells of microtiter plate. The plates were incubated for 2 hrs at room temperature and then at 4°C overnight. The wells were washed 3 times with ST-20 and unoccupied sites were filled with 1.0 percent BSA in ST-20 by incubating the plate at 4°C overnight. After washing the plate with ST-20, 0.25 ml of serially diluted serum in ST-20 was added. The plates were incubated for 1 hr at 37°C, washed with ST-20 and 0.25 ml of thousand time diluted anti-rabbit IgG alkaline phosphatase conjugate was added. At the end of 2 hrs incubation at 37°C, the plate was emptied and washed with ST-20 thrice followed by one washing with distilled water. Substrate solution (PNP -P, 0.25 ml) was added. The plate was incubated at 37°C for 1 hr and the reaction was stopped by the addition of 0.05 ml of 3N NaOH. The contents of each well were

read at 400 nm. Results were expressed as $A_{\text{test}} - A_{\text{control}}$.

14. Immunohistological Studies

(1) Immunization of rats

Male rats (weighing 50-60 gm) were injected intraperitoneally with 100 μ g of native/irradiated hCG on alternative days. After 4 injections the animals were sacrificed and testes were removed.

(ii) Histology

For histological studies, testes were fixed in Bouin's (alcoholic) solution for 24 hrs and washed several times with 70% alcohol. These were later taken through different grades of alcohol and cleared in xylene, infiltrated with 50:50 of wax and xylene and finally embedded in pure paraffin (50-60°C). Section of 5-7 μ thickness were cut by rotary microtome and stained with hematoxylin and counter stained with eosin. The stained sections were dehydrated and cleared in usual manner and mounted in DPX.

15. Dark Binding of Psoralen to hCG

Fluorescence intensity of psoralen was studied in 0.05M phosphate buffer, pH 7.4 containing 0.9 percent sodium chloride (Veronese et al, 1979). The excitation and emission wavelengths of psoralen obtained from the fluorescence spectra were found to be 330 nm and 450 nm respectively. The interaction of

psoralen with hCG was investigated by monitoring its intrinsic fluorescence in free and bound (hCG-psoralen complex) states after incubating it in dark for 24 hours at room temperature. The difference in fluorescence intensities of the drug in the above two states (fluorescence quenching) was assumed to be proportional to the amount of hCG bound drug.

16. Fluorescence Transition of hCG in 8M Urea

0.5 ml of hCG (1 mg/ml in 0.05M phosphate buffer, pH 7.4) and 2.0 ml of 10M urea were mixed to give a final concentration of hCG as 200 μ g/ml and that of urea 8M. Fluorescence intensity was recorded at 360 nm (excitation wavelength 280 nm). The fraction of hCG in denatured state was calculated using the following expression (Tanford, 1968),

$$f_D = \frac{(F)_{\text{obs}} - (F)_N}{(F)_D - (F)_N}$$

where $(F)_N$, $(F)_{\text{obs}}$ and $(F)_D$ represent the fluorescence intensities in native, observed and denatured states respectively.

17. Gel Filtration

Dark binding of psoralen to hCG was also studied by gel filtration chromatography. Native hCG and hCG-psoralen complex were chromatographed on Sephadex G-100 column (1.8 cm x 66.0 cm) and eluted with 0.05M phosphate buffer, pH 7.4 containing 0.9% NaCl. 2.0 ml fractions were collected at a flow rate of 18 ml/hr.

18. Immunization

Rabbits were immunized with the complex of psoralen with hCG and its α and β -subunits suspended in Freund's complete adjuvant by intramuscular injection weekly for three weeks. Blood was collected at weekly intervals by heart puncture. Sera were separated by centrifugation and stored at -4°C until use.

19. Antibody Evaluation in Sera by ELISA

Preimmunized and immunized rabbit sera at different dilutions were tested by ELISA. Wells of polystyrene plate were coated with hCG ($1\mu\text{g}/\text{well}$ in coating buffer) as described earlier. The reactivity of induced antibodies was checked by making a series of serum dilutions. The enzyme activity in conjugate was monitored with p-nitrophenyl phosphate at 400 nm and results were expressed as $A_{\text{test}} - A_{\text{control}}$.

20. Determination of Protein Concentration

Protein was estimated by the method of Lowry et al (1951) as described below:

(a) Preparation of Folin-phenol reagent

The Folin-phenol reagent was prepared by the procedure given by Folin and Ciocalteu (1927). 100 gm of sodium tungstate, 25 gm of sodium molybdate, 700 ml of distilled water, 48.2 ml of 85 percent orthophosphoric acid and 100 ml of hydrochloric acid were refluxed in dark for 10 hrs. 128 gm of lithium sulfate,

50 ml of distilled water and few drops of bromine were added to the mixture. Excess bromine was removed by boiling the mixture for 15 minutes. The solution was cooled, diluted to one liter and filtered. The bright yellow reagent was protected from light by storing it in amber colored bottle. The above reagent was diluted four times with distilled water before use.

(b) Alkaline copper reagent

The components of alkaline copper reagent were as follows:

- (i) 2 percent sodium carbonate in 0.01M sodium hydroxide.
- (ii) 0.5 percent copper sulfate in 1 percent sodium potassium tartarate.

50 ml of (i) was mixed with 1.0 ml of (ii). The reagent was prepared fresh before use.

(iii) Procedure

1.0 ml of protein sample was mixed with 5.0 ml of alkaline copper reagent. The tubes were kept for 10 minutes at room temperature and 1.0 ml of diluted Folin-phenol reagent was added. The contents were mixed immediately and allowed to stand for 30 minutes at room temperature. The absorbance was read at 660 nm. Concentration of unknown samples were computed from a standard plot drawn by using bovine serum albumin.

21. Isolation of IgG

IgG from preimmune and immune sera was isolated by DEAE cellulose adsorption method of Stanworth (1960). IgG was isolated

also from crude immunoglobulins by DEAE cellulose chromatography.

(a) Isolation of IgG by DEAE cellulose adsorption method

Approximately 17.0 gm of moist DEAE cellulose previously washed and equilibrated with 0.01M sodium phosphate buffer containing 0.015M NaCl, pH 7.5 was mixed with 2.5 ml of cold phosphate buffer. 2.5 ml of serum (previously dialyzed for 18 hr at 4°C against phosphate buffer) was added dropwise to the exchanger with continuous mixing. The mixture was kept for 5 hr at 4°C. Thereafter, 5.0 ml of cold buffer was added with stirring. The resulting slurry was centrifuged in cold for 15-20 minutes. The supernatant containing IgG was decanted and cleared from cellulose, if any, by recentrifugation. The purity of the isolated IgG was checked by polyacrylamide gel electrophoresis using 10 percent gel.

(b) Isolation of IgG by DEAE Cellulose Chromatography

(1) Preparation of crude immunoglobulins

3.0 ml of 100 percent saturated ammonium sulfate solution was slowly added to 5.0 ml of serum in cold with occasional shaking and the mixture was kept at 4°C for 2 hr for complete precipitation of the immunoglobulins. The precipitate was recovered by centrifugation and washed thrice with 37 percent saturated ammonium sulfate solution. The washed precipitate was dissolved and dialyzed against 0.01M phosphate buffer, pH 8.0

(11) DEAE cellulose chromatography

Dialyzed crude immunoglobulins were loaded onto a column of DEAE cellulose (2cm x 20cm) previously equilibrated with starting buffer (0.01M phosphate buffer, pH 8.0). The column was eluted at room temperature with linear ionic strength gradient of 0.01M-0.3M phosphate buffer, pH 8.0. 3.0 ml fractions were collected and monitored for protein (Fig.4). First peak of the chromatogram was pooled and used as purified IgG.

22. Binding of Psoralen to hCG by ELISA

Plates were coated with hCG (1 μ g/well in bicarbonate buffer) overnight at 4°C. Wells were washed with ST-20 and unoccupied sites were filled with BSA (1% in ST-20). Psoralen (10 ng to 100 ng in ST-20) was added prior of the addition of antibodies. After addition of psoralen, plate was incubated in dark for 24 hours. ELISA was performed as described earlier.

23. Interaction of Psoralen to hCG in Presence of UV-A Light

Ultraviolet irradiation was carried out at room temperature. A long wavelength ultraviolet lamp having maximum emission at 365 nm was used as the source of radiant energy. 3.0 ml aqueous solution of hCG (100 μ g/ml) in 0.05M phosphate buffer containing 0.9% NaCl, pH 7.4 in the presence of psoralen (2-10 μ g/ml) was irradiated at a distance of 7.0 cm from the lamp for different time periods. After irradiation, their fluorescence intensities were recorded at emission, maxima of psoralen.

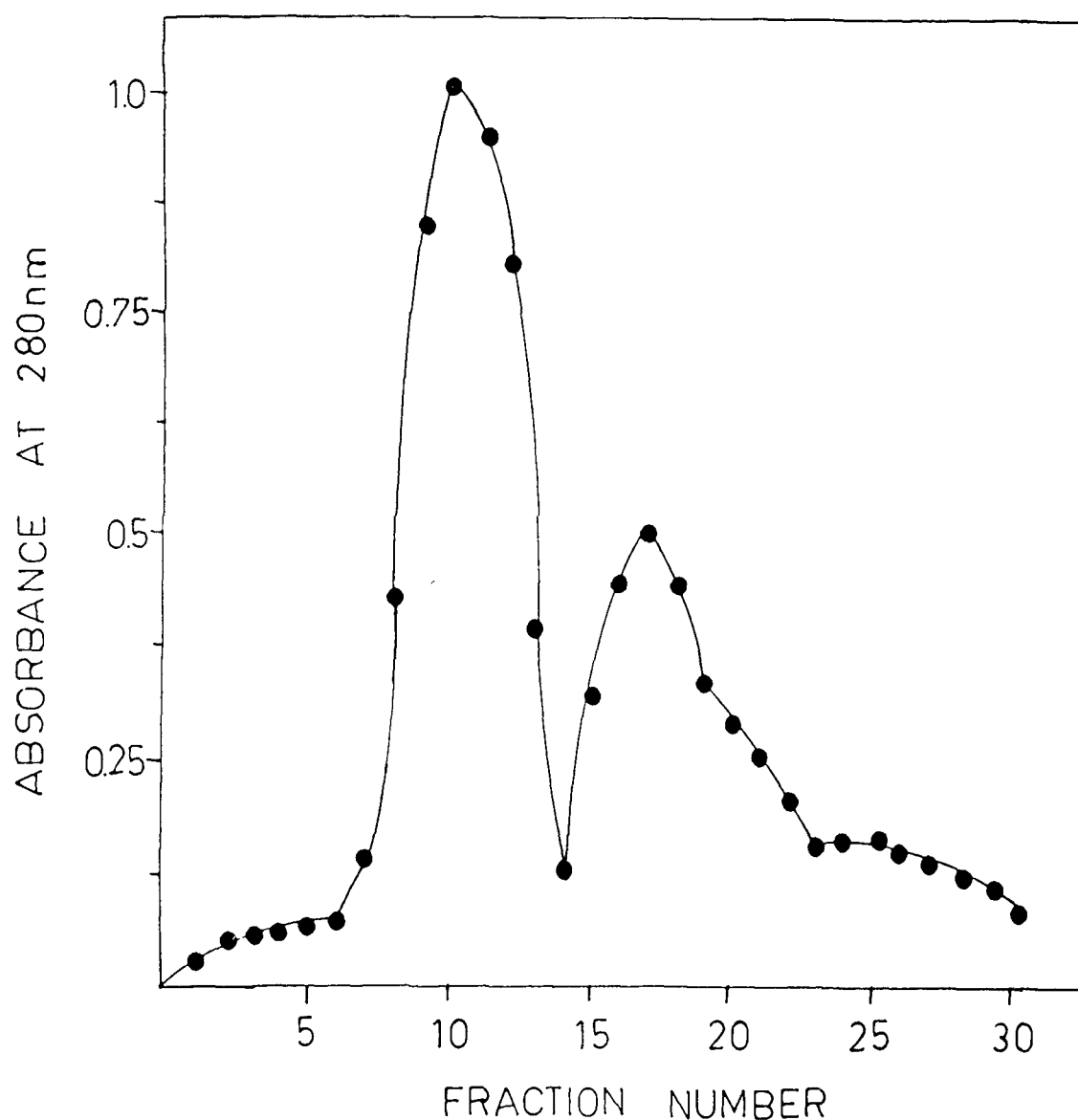


Fig.4. Isolation of serum IgG by DEAE cellulose chromatography.

Thirty seven percent saturated ammonium sulfate precipitated serum fraction was used for isolation of IgG. The elution was performed by applying a linear ionic gradient of 0.01M - 0.3M phosphate buffer, pH 8.0. Fractions of 3.0 ml were collected and monitored for protein.

24. Dark Interaction of Psoralen to Alpha and Beta Subunits of hCG

Fluorescence spectral properties of hCG subunits and psoralen were studied in 0.05M phosphate buffer, pH 7.4 containing 0.9 percent NaCl. The extent of interaction between psoralen and subunits of hCG was evaluated as described earlier. Subunits binding to psoralen was also studied by gel filtration on Sephadex G-100 as described earlier.

25. Chymotrypsin Digestion of Beta-Subunit

Chymotrypsin digestion of β -subunit was done by the method of Carlsen et al (1973). Briefly the method was as follows:

(i) Carboxymethylation of β -hCG

To 10 mg of β -subunit in 3.0 ml of 8M urea, 0.5M Tris and 0.001M EDTA, pH 8.1, was added 30 mg dithiothreitol. The mixture was incubated at 37°C for 4 hr, after which 0.1 ml of iodoacetic acid (72.0 mg in 0.5N NaOH) was added and pH was maintained at 8.0 with 0.5N NaOH. The reaction mixture was stirred in dark for 30 minutes.

(ii) Hydrolysis with chymotrypsin and gel filtration

The incubated material was digested with chymotrypsin for 4 hr at 37°C and subjected to gel filtration through Sephadex G-25 column previously equilibrated with 0.5M sodium bicarbonate containing 0.02 percent sodium azide.

(iii) Purification of peptides of β -subunit

Further purification of peptides of β -hCG was done on Sephadex G-50 column. Each subunit in PBS containing 8M urea and 5 mM EDTA was loaded onto a column of Sephadex G-50 (1.8cm x 55cm) previously equilibrated with PBS. Fractions (2.5 ml) were collected at a flow rate of 20 ml/hr.

26. Isolation and Purification of Anti-Peptide Antibodies from Anti-hCG Immune Sera

Anti-peptide antibodies were isolated from anti-hCG immune sera by affinity chromatography.

(i) Preparation of peptide-Sepharose 4B column

Sepharose 4B gel was washed with distilled water on sintered glass funnel. 5 gm of moist gel was mixed with 20 ml of 2M Na_2CO_3 at 4°C . One gm of CNBr was dissolved in 0.6 ml of acetonitrile and kept at 4°C . CNBr solution was added dropwise to the mixture and contents were mixed for 15-20 minutes in cold. The mixture was filtered through sintered glass funnel kept at -20°C and washed with 0.1M Na_2CO_3 containing 0.5M NaCl (coupling buffer). The gel was then transferred to a stoppered cylinder containing 8-10 mg of peptide. The contents were mixed (end-over-end) at room temperature for 2 hr and then kept at 4°C for 14 hrs.

The coupled gel was mixed again for 1 hr at room temperature and thereafter filtered on the sintered glass funnel and washed

with approximately 200 ml of 0.1M NaHCO_3 . After washing with 1M ethanolamine the slurry was transferred to stoppered cylinder containing approximately 50 ml of 1M ethanolamine to block unoccupied sites. The gel was mixed (end-over-end) for 2 hrs at room temperature, filtered and washed with 200 ml of 0.1M NaHCO_3 and then with 200 ml of 0.1M acetate buffer, pH 4.0 and finally with PBS for equilibration.

(ii) Purification of anti-peptide antibodies by affinity chromatography

A solution of 10 mg/ml of crude gammaglobulins of anti-hCG was dialyzed in PBS and applied onto affinity column (3cm x 6cm). The column was washed with PBS to remove unbound material. The bound material was eluted with 6M urea in PBS. The eluate was immediately dialyzed against PBS to remove urea and concentrated.

R E S U L T S

1. Purification of Human Chorionic Gonadotropin (hCG)

Commercially available hCG was purified by gel filtration chromatography on Sephadex G-100 in 0.05M phosphate buffer, pH 7.5 (Fig.5). The hormone sample was contaminated with subunits and unbound sugar contents. The first major peak was intact hCG whereas other peaks were due to microheterogeneity of the hormone molecule. The fractions of first major peak were pooled, concentrated and again loaded onto same filtration medium. The first major peak (Fig.6) was pooled and used in subsequent studies.

2. Polyacrylamide Gel Electrophoresis (PAGE)

Commercially available and purified hormone samples were subjected to polyacrylamide gel electrophoresis. The impure hCG showed heterogeneity (Fig.7A) whereas the purified hormone was found to be homogeneous with respect to charge and exhibited a single electrophoretic band (Fig.7B).

The subunit composition of purified hCG was determined by SDS PAGE (Fig.8). The sample was found constituting two subunits designated as alpha subunit (α , low molecular weight) and beta subunit (β , high molecular weight). The omission of β -mercaptoethanol had no effect on electrophoretic mobility of subunits, suggesting that these subunits are held together by non-covalent bonds.

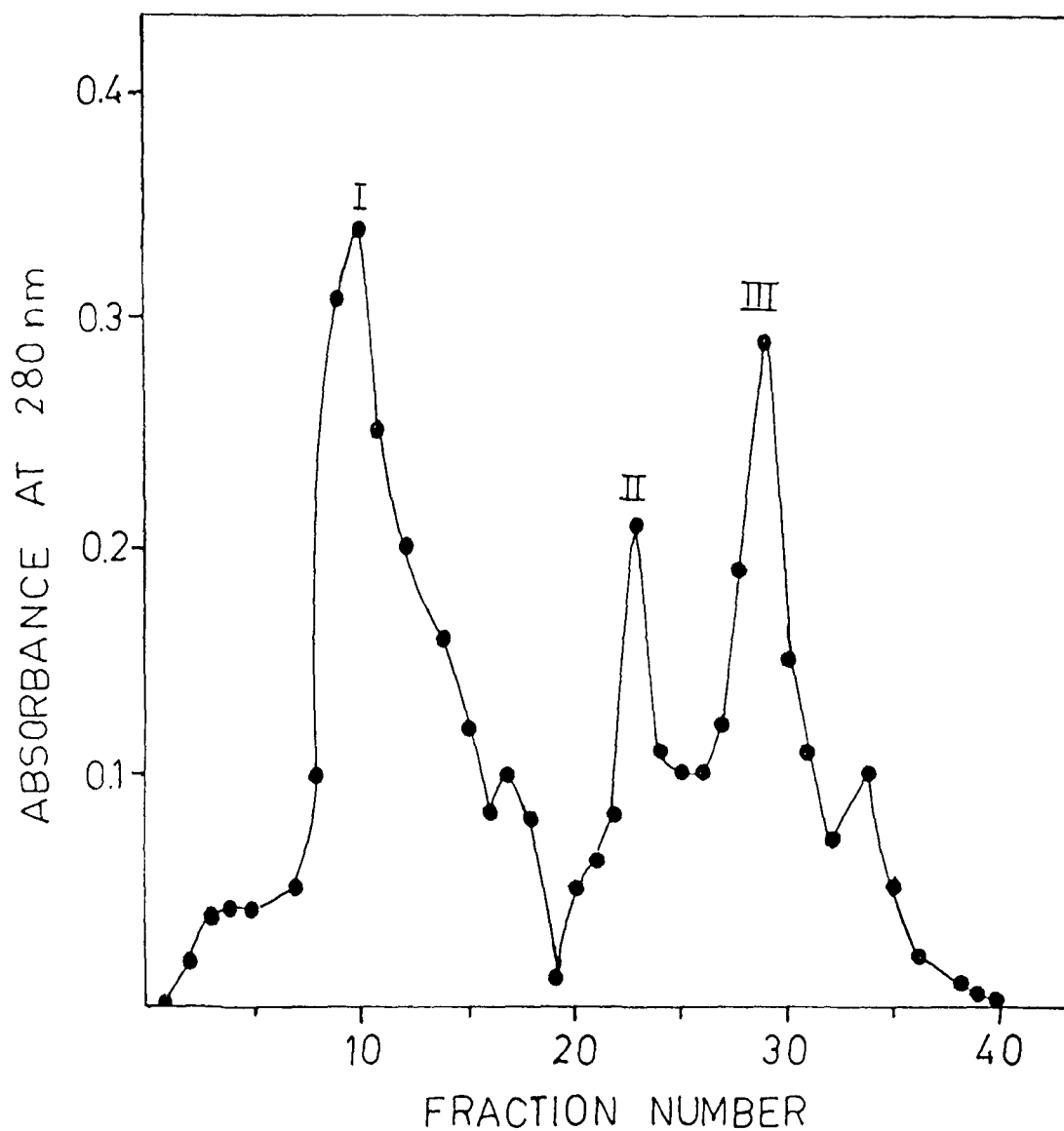


Fig.5. Gel filtration of commercial hCG on Sephadex G 100 column.

Column size : 1.8 x 66.0cm; eluent : 0.05M phosphate buffer, pH 7.5; fraction size : 2.5 ml; flow rate: 18 ml/hr. Fraction under peak I were pooled, dialyzed against 0.05M phosphate buffer pH 7.5 and again loaded to the same column.

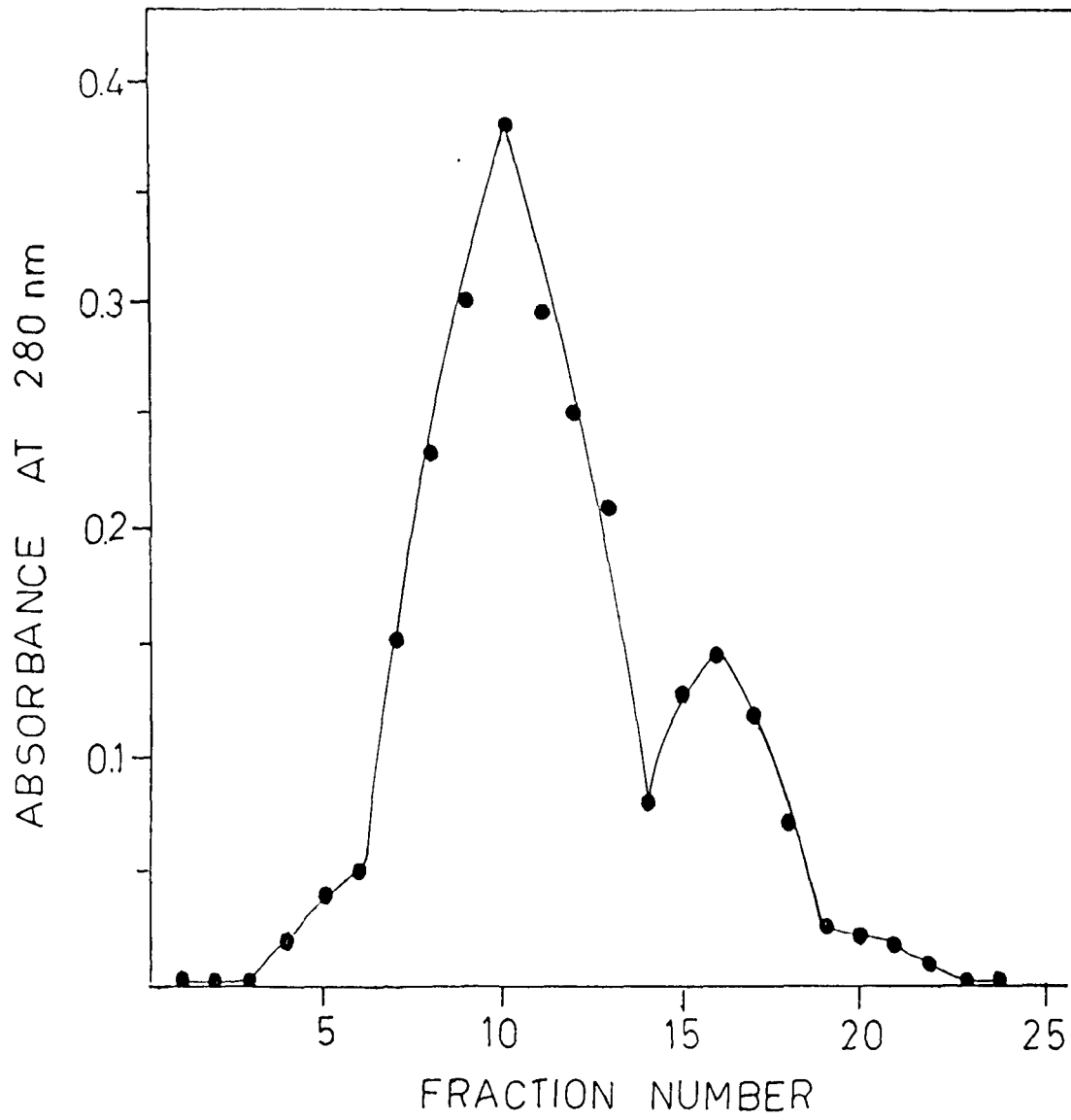


Fig. 6. Elution profile of purified hCG on Sephadex G 100 column.

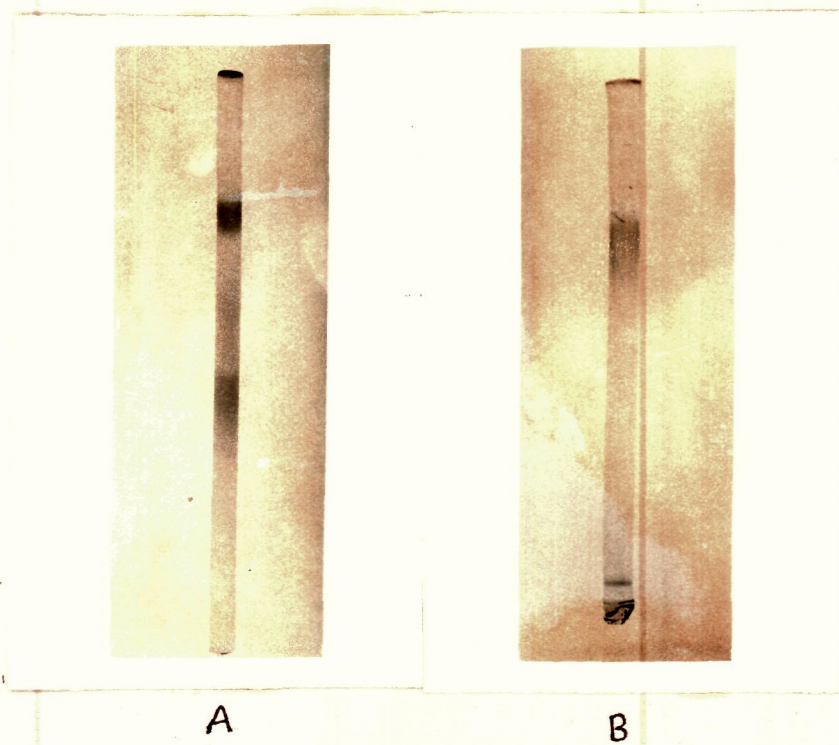


Fig.7. Polyacrylamide gel electrophoretic pattern of commercial and purified hCG.

A = commercial hCG; B = purified hCG.

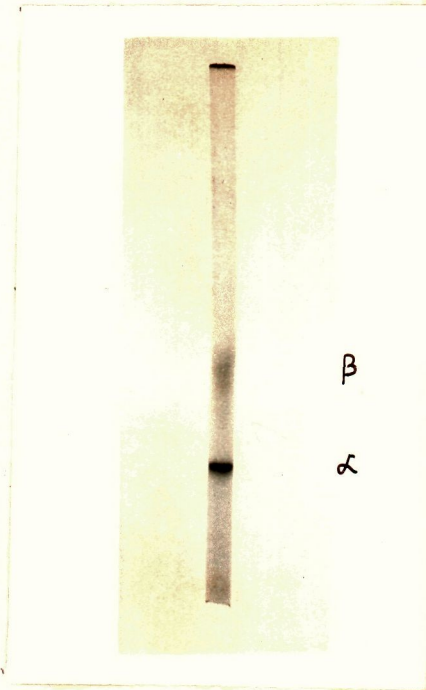


Fig.8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of hCG.

100 μ g of hCG was electrophoresed in 10 percent polyacrylamide gel containing 0.1 percent SDS. The gel was stained with Coomassie Blue. α and β represents the alpha and beta-subunits of hCG.

3. Effect of UV Radiation on hCG

(i) Fluorescence difference spectra

The fluorescence spectral properties of hCG were studied at pH 7.5. The native hormone exhibited a single emission maximum at 350 nm when excited with 280 nm (Fig.9). On irradiation with different UV doses of wavelength 254 nm, the hormone showed a dose dependent loss in intrinsic fluorescence intensity. The fluorescence difference spectra were constructed by subtracting fluorescence intensity values of irradiated hCG from those of native hCG at various emission wavelengths (Fig.10). The difference spectra exhibited two emission maxima at 350 nm and 410 nm. The first one is the characteristic maxima of protein but the second maxima might be due to the scattering of light.

(ii) Ultraviolet spectra

UV absorption spectra of native and irradiated hCG were recorded in the spectral range from 230 nm to 400 nm. Both native and irradiated hCG showed a maximum at 275 nm and minimum at 250 nm (Fig.11). The irradiated hCG exhibited decreased absorption over entire spectral range and the decrease at 275 nm was proportional to radiation dose (Table 3).

4. Modification of Lysine Residues of hCG

2,4,6 trinitrobenzene sulfonic acid (TNBS) which is generally considered to be an amino group specific reagent (Ogiso et al, 1974; Ernest and Kim, 1974) was used to modify

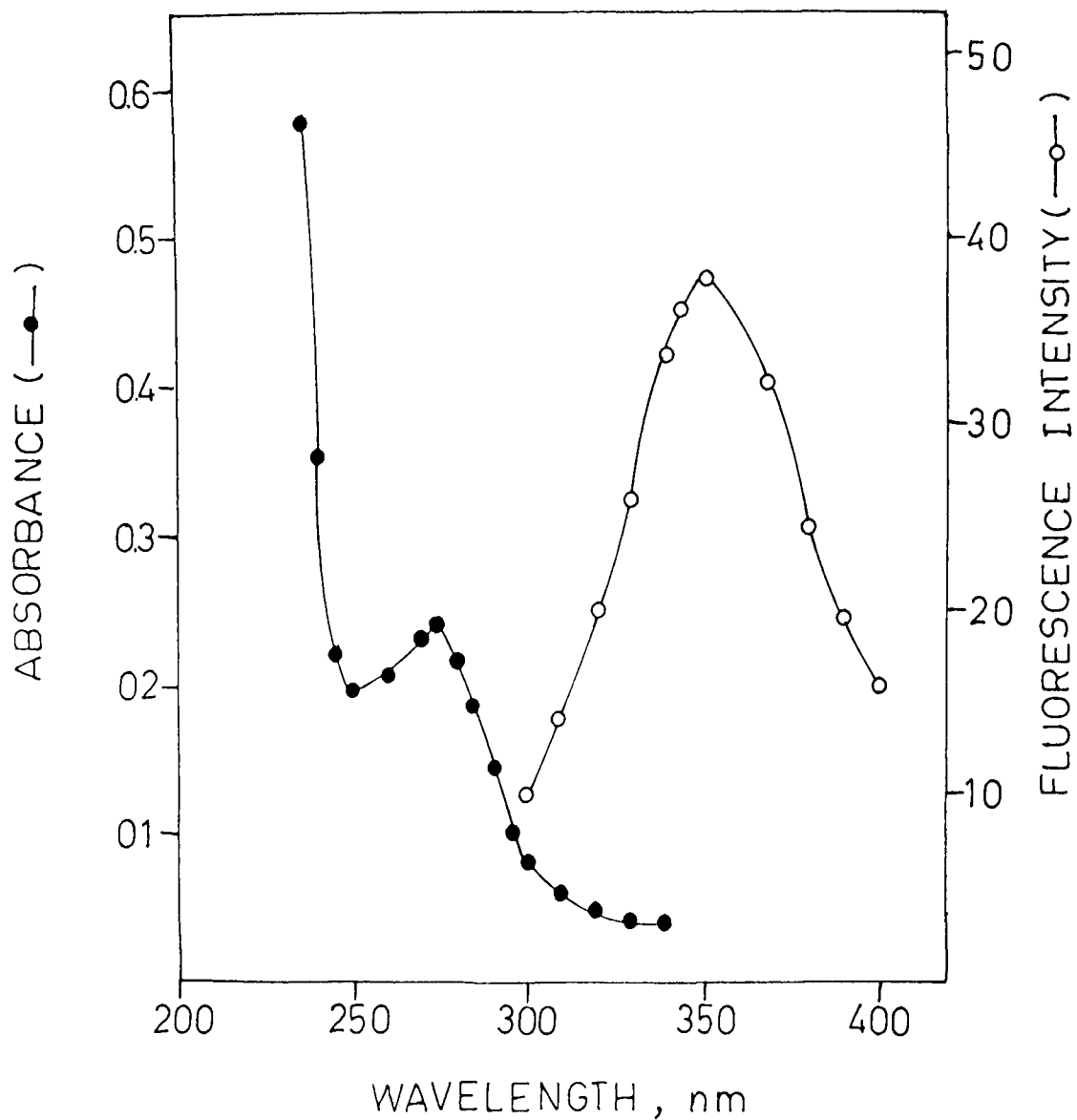


Fig.9. Ultraviolet and fluorescence spectra of native hCG.

Hormone (500 $\mu\text{g/ml}$) was dissolved in phosphate buffer, pH 7.5. Ultraviolet (—●—) and fluorescence spectra (—○—).

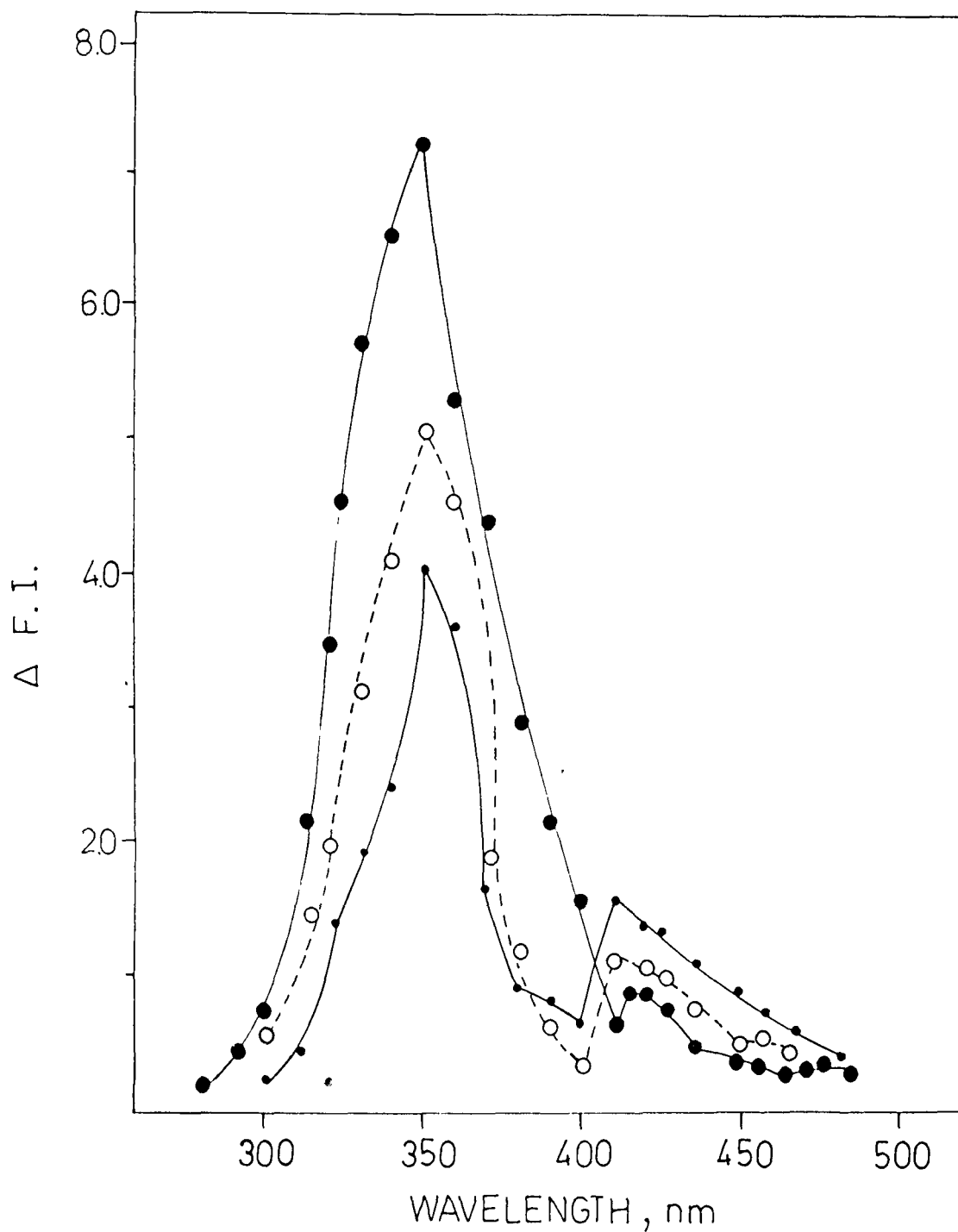
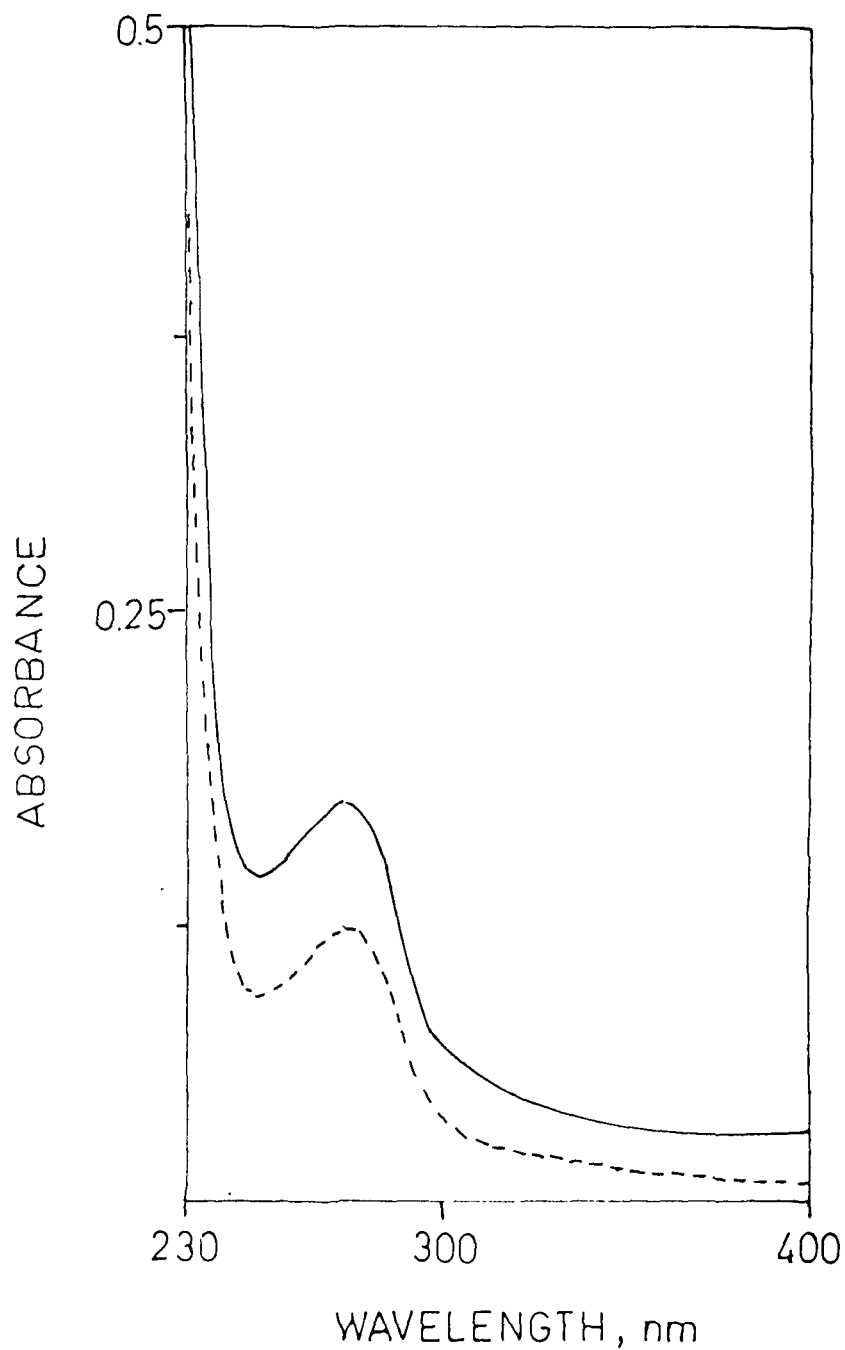


Fig.10. Fluorescence difference spectra of photoinactivated hCG.

The hormone (500 $\mu\text{g/ml}$) was in 0.05M phosphate buffer, pH 7.5. Radiation doses $18.2 \times 10^7 \text{ ergs cm}^{-2}$ (—●—); $36.4 \times 10^7 \text{ ergs cm}^{-2}$ (---○---) and $54.6 \times 10^7 \text{ ergs cm}^{-2}$ (—●—).



, Fig.11. Ultraviolet spectra of photoinactivated hCG.

Hormone (500 $\mu\text{g}/\text{ml}$) was in phosphate buffer, pH 7.5. Native (—) and 18.2×10^{-7} ergs cm^{-2} irradiated hCG (- - - - -).

TABLE III

Ultraviolet absorption at 275 nm of native and irradiated hCG

Radiation dose $\times 10^7$ ergs cm^{-2}	Absorbance at 275 nm	
	Absorbance	Percent decrease
Native	0.508	-
18.2	0.312	38.58
36.4	0.283	44.29
54.6	0.274	46.06

free lysine amino groups of hCG. The kinetics of the lysine residue modification was monitored. Total number of lysine residues of hCG have been reported to be 10 (Morgan et al, 1975). In the present studies, the trinitrophenylation of only 6 lysine residues (Fig.12) in native hCG suggests that the remaining four lysine residues are buried in the molecule and thus inaccessible to the reagent. The irradiated (18.2×10^7 ergs cm^{-2}) hCG showed 5 trinitrophenylated lysine residues. On two fold increase in the radiation dose, almost no effect was observed in the number of lysine residue modified.

5. Tryptic Digestibility of hCG

The susceptibility of native and irradiated hCG to tryptic digestion was checked in terms of number of moles of additional amino groups exposed after proteolytic digestion. The results (Fig.13) showed that native hCG which is a compact structure (Ingham et al, 1976) is completely resistant to tryptic attack even after its incubation with trypsin for 2 hrs. However, after irradiation at a dose of 18.2×10^7 ergs cm^{-2} hCG became susceptible to tryptic attack. With increased radiation doses, the susceptibility to trypsin was found decreased.

6. Effect of Radiation on Reducing Sugars

Reducing sugars were estimated in native and irradiated hCG samples (Fig.14). Their contents were found increased with radiation dose.

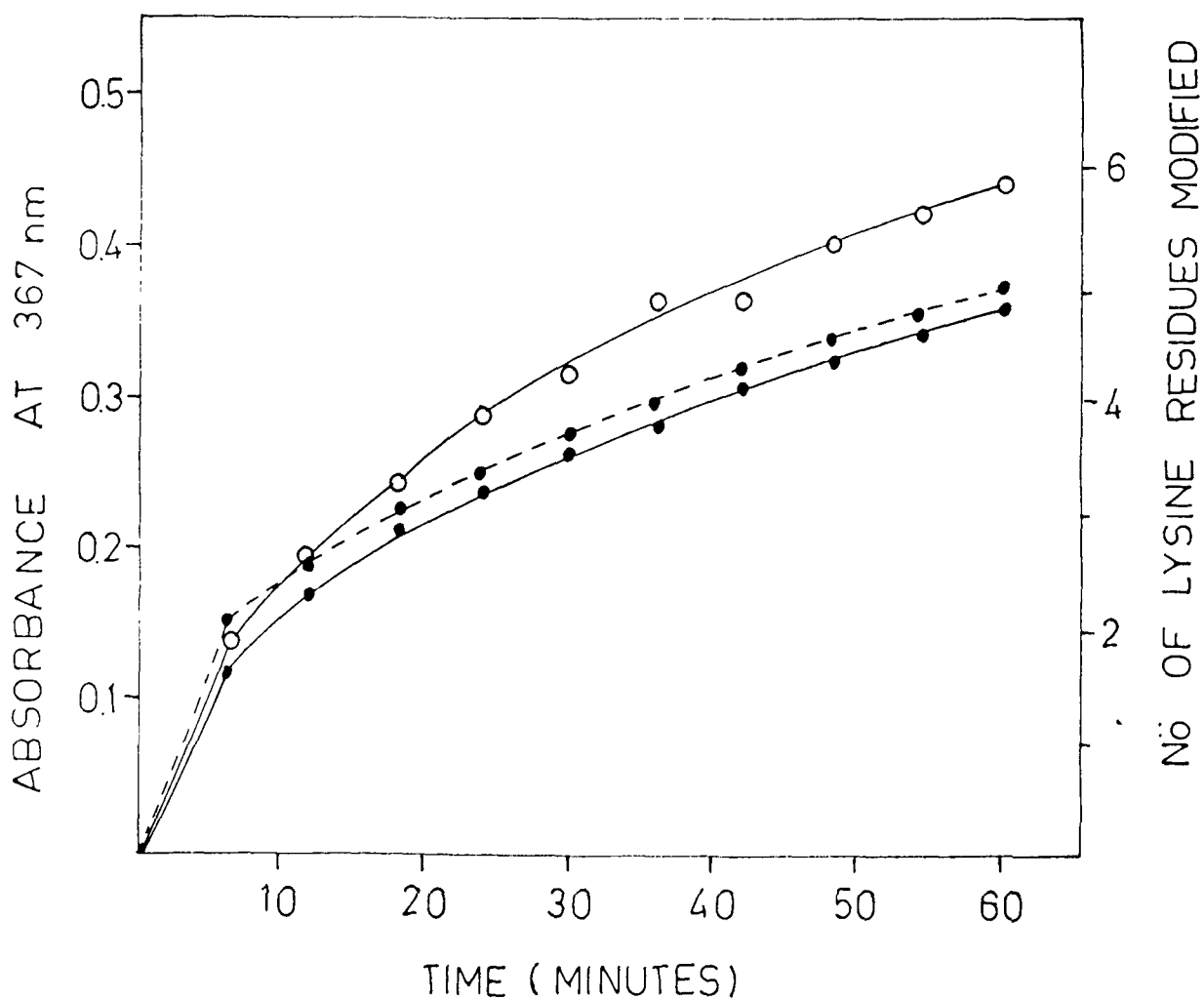


Fig.12. Kinetics of modification of lysine residues of native and irradiated hCG with 2,4,6-trinitrobenzene sulfonic acid (TNBS).

Native hCG (—○—); 18.2×10^7 ergs cm^{-2} ,
 irradiated hCG (---●---) and 36.4×10^7 ergs cm^{-2}
 irradiated hCG (—●—).

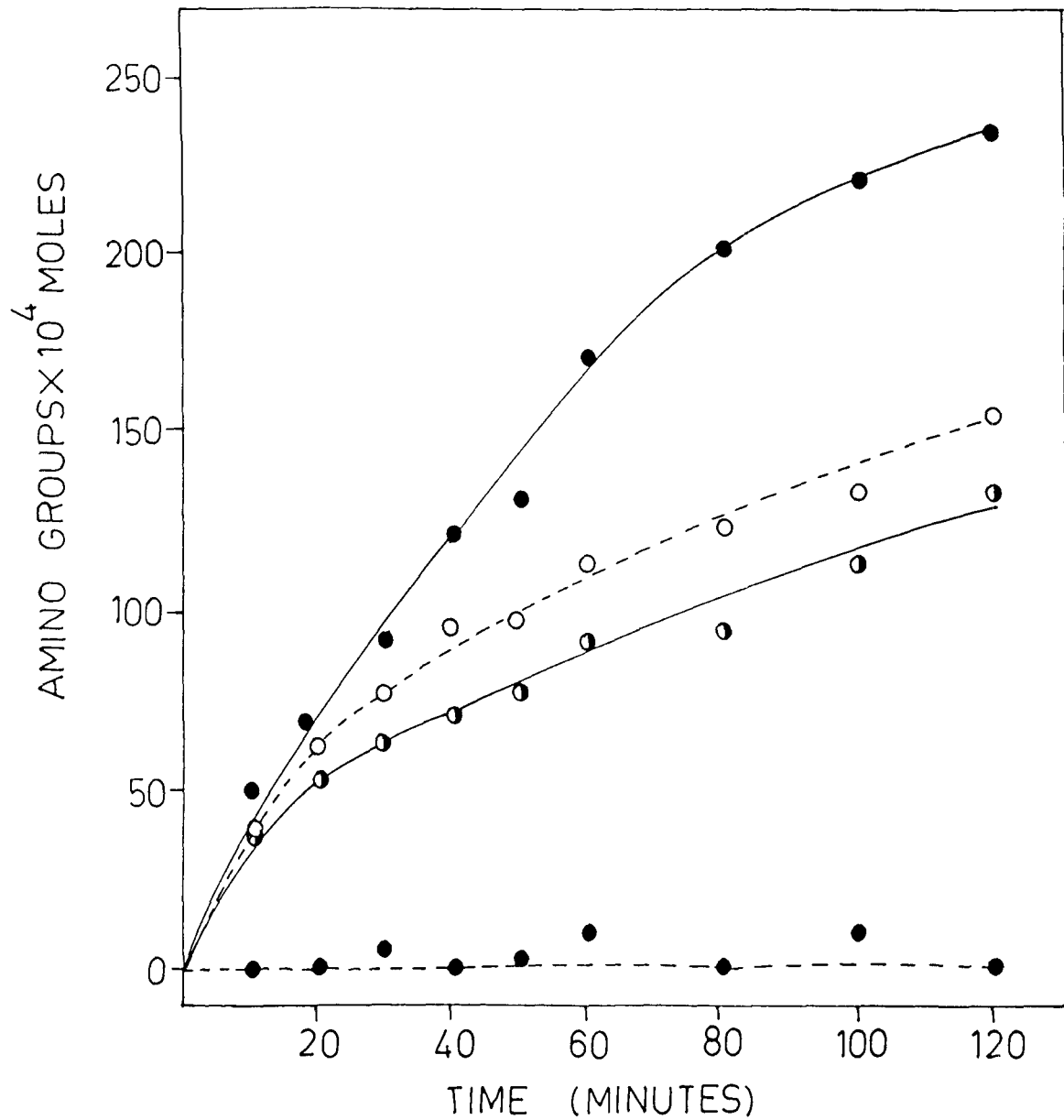


Fig.13. Effect of ultraviolet irradiation on tryptic digestibility of hCG.

Native hCG (---●---); 18.2×10^7 ergs cm^{-2}
 (—●—) 36.4×10^7 ergs cm^{-2} (---○---) and
 54.6×10^7 ergs cm^{-2} (—○—) irradiated hCG.

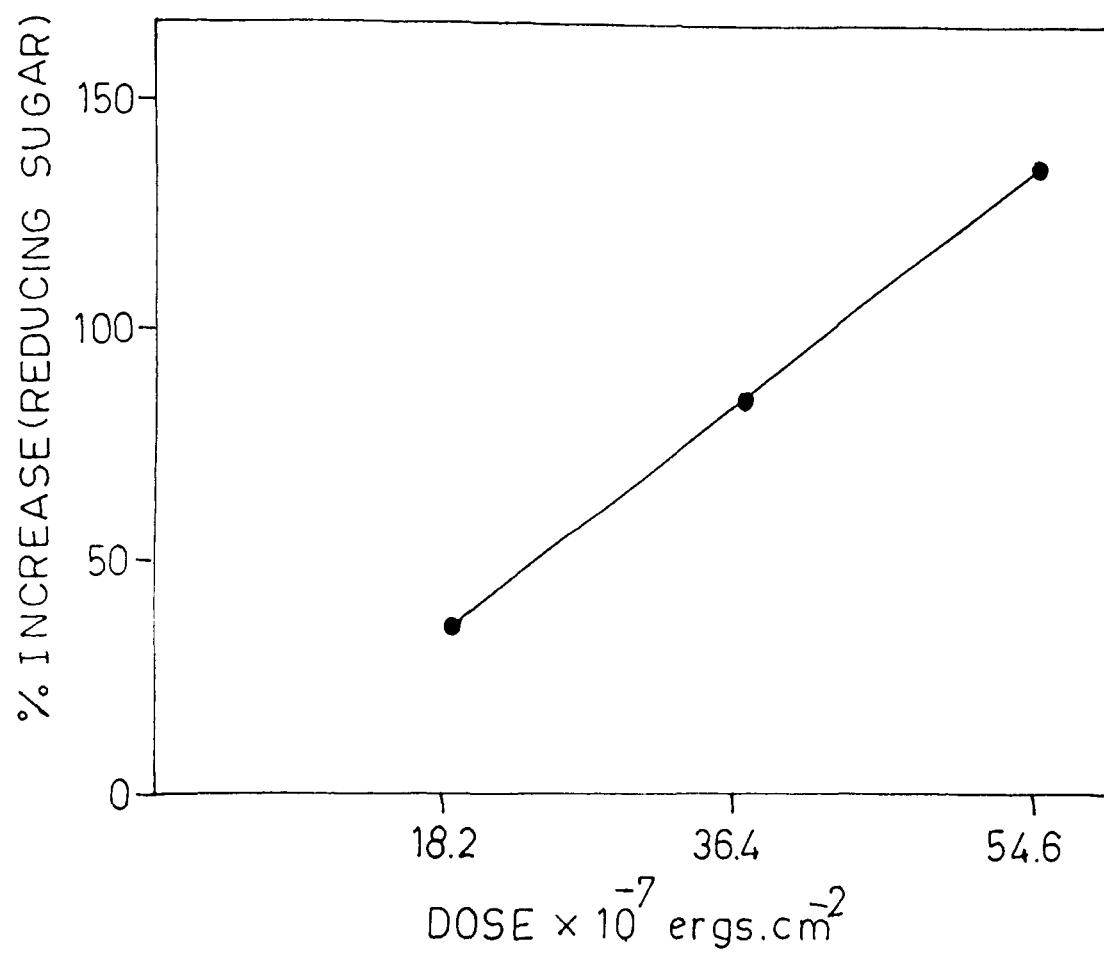


Fig.14. Effect of irradiation on reducing sugars.

7. Unfolding of hCG by Urea

The fluorescence intensity of hCG at 350 nm is due to the presence of aromatic amino acids. Perturbation of the environment of these amino acid by treatment of the hormone with a denaturant is expected to alter its intrinsic fluorescence. Therefore, urea denaturation of hCG was followed by monitoring the loss in its intrinsic fluorescence at 350 nm during unfolding process.

The concentration range of urea over which the native hCG was denatured is typical for globular proteins (Tanford, 1968). The hormone resists unfolding only upto 1 M urea (Fig.15), but as the urea concentration is increased, it is abruptly denatured suggesting that subunits of hCG are held together with non-covalent bonds and denaturation is a two state process i.e. native \rightleftharpoons denatured. Relatively higher concentration of urea was needed for complete unfolding of the molecule. A significant change in the unfolding behaviour of irradiated hCG was noticed.

8. Antibody Activity Measurements in Immune Sera

Antibodies were raised in rabbits both against native and irradiated hCG (18.2×10^7 ergs cm^{-2}) with Freund's complete adjuvant. The level of induced antibodies was checked by counterimmunoelectrophoresis (CIE) and ELISA on polystyrene plates. Precipitating antibodies were obtained with hCG as antigen (Fig.16). The antibody recognized irradiated hCG but to

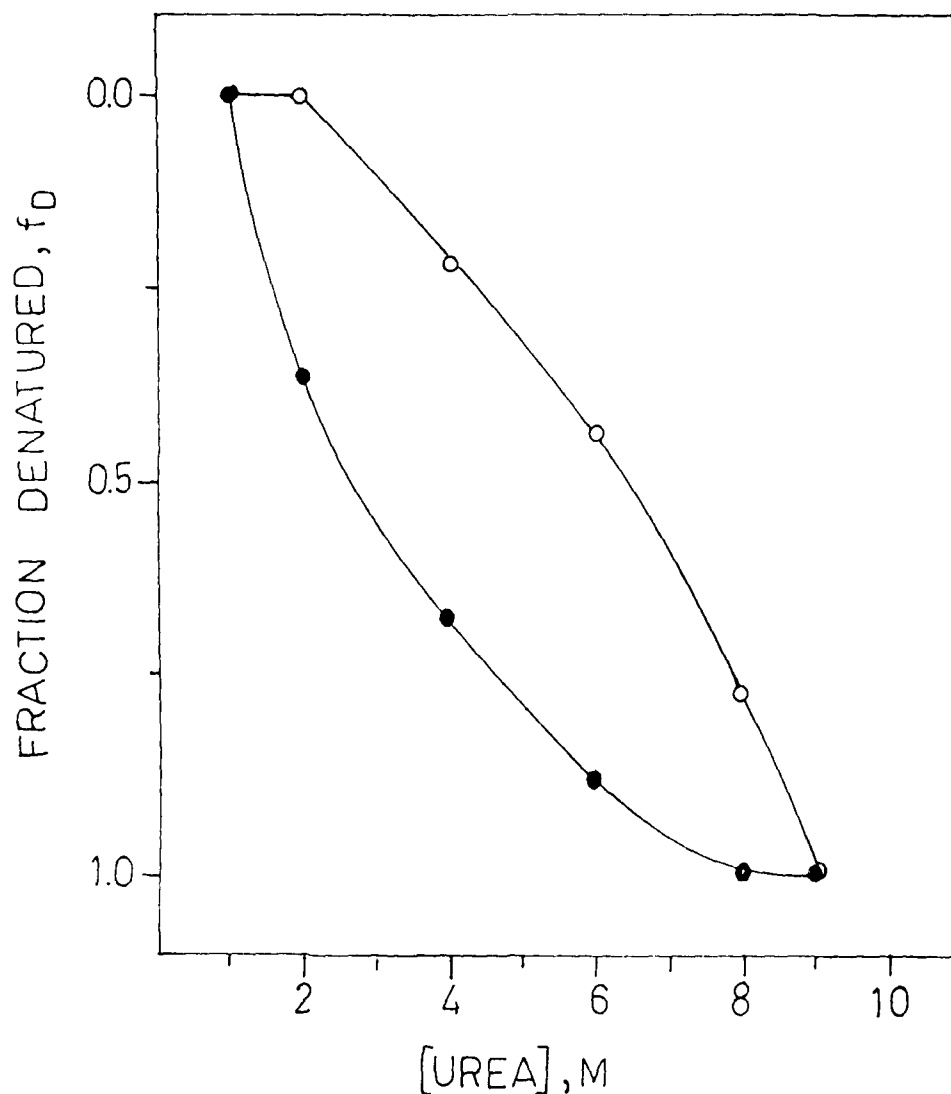
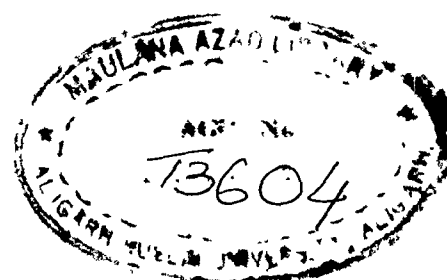


Fig.15. Urea denaturation profiles of native and irradiated hCG.

Native (—●—) and irradiated hCG at $18.2 \times 10^7 \text{ ergs cm}^{-2}$ dose (—○—). The value of f_D were calculated as described in text. hCG conc. 500 $\mu\text{g/ml}$ phosphate buffer, pH 7.5.



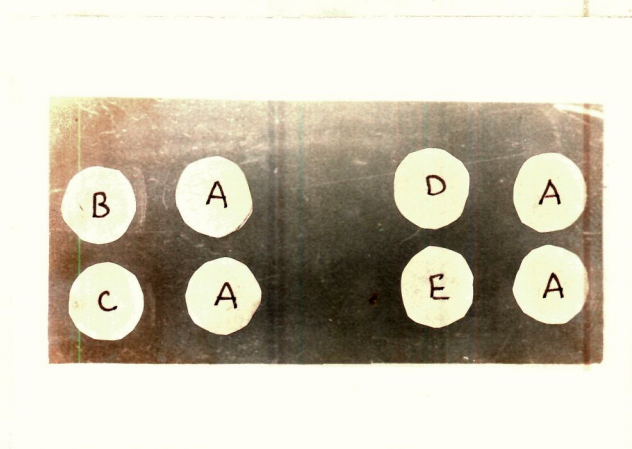


Fig.16. Antibodies against hCG detected by CIE.

A = anti-hCG immune sera, B = native hCG,
C = 18.2×10^7 ergs. cm^{-2} irradiated hCG,
D = 36.4×10^7 ergs cm^{-2} irradiated hCG and
E = 54.6×10^7 ergs cm^{-2} irradiated hCG.

73

a lower extent. At higher irradiation doses, the hCG fail to form precipitation lines. Direct binding ELISA of antisera against irradiated hCG (Fig.17) showed increased antibody level with increased period of immunization. The absorbance of less than 0.1 was taken as cut off value for the computation of serum titer. The titer of antisera was found to be 1:0, 1:100, 1:400 and 1:1600 after first, second, third and fourth injections respectively. The results also indicate that a minimum of two injections are required for detectable antibody activity.

9. Immunohistology of Rat Testes

In case of control rats, testis showed normal seminiferous tubules and spermatogenesis. However, when hCG was injected into the rat, the testis showed seminiferous tubules with increased spermatogenesis. The tubules were filled fully with mature sperms. While in case of irradiated hCG injected rat, testis showed normal seminiferous tubules with inhibited maturation, suggesting that normal sperm formation did not take place. Few sertoli cells were also damaged and a decrease in their number was evident (shown by arrow, Fig.18).

10. Dark Binding of Psoralen to hCG

Information on the binding between psoralen and hCG was first obtained by equilibrium dialysis. With $1.35 \times 10^{-6} \text{ M}$ hormone in 0.05 M phosphate buffer, pH 7.4 containing 0.9% NaCl and $5.38 \times 10^{-5} \text{ M}$ psoralen at room temperature, over 32 percent

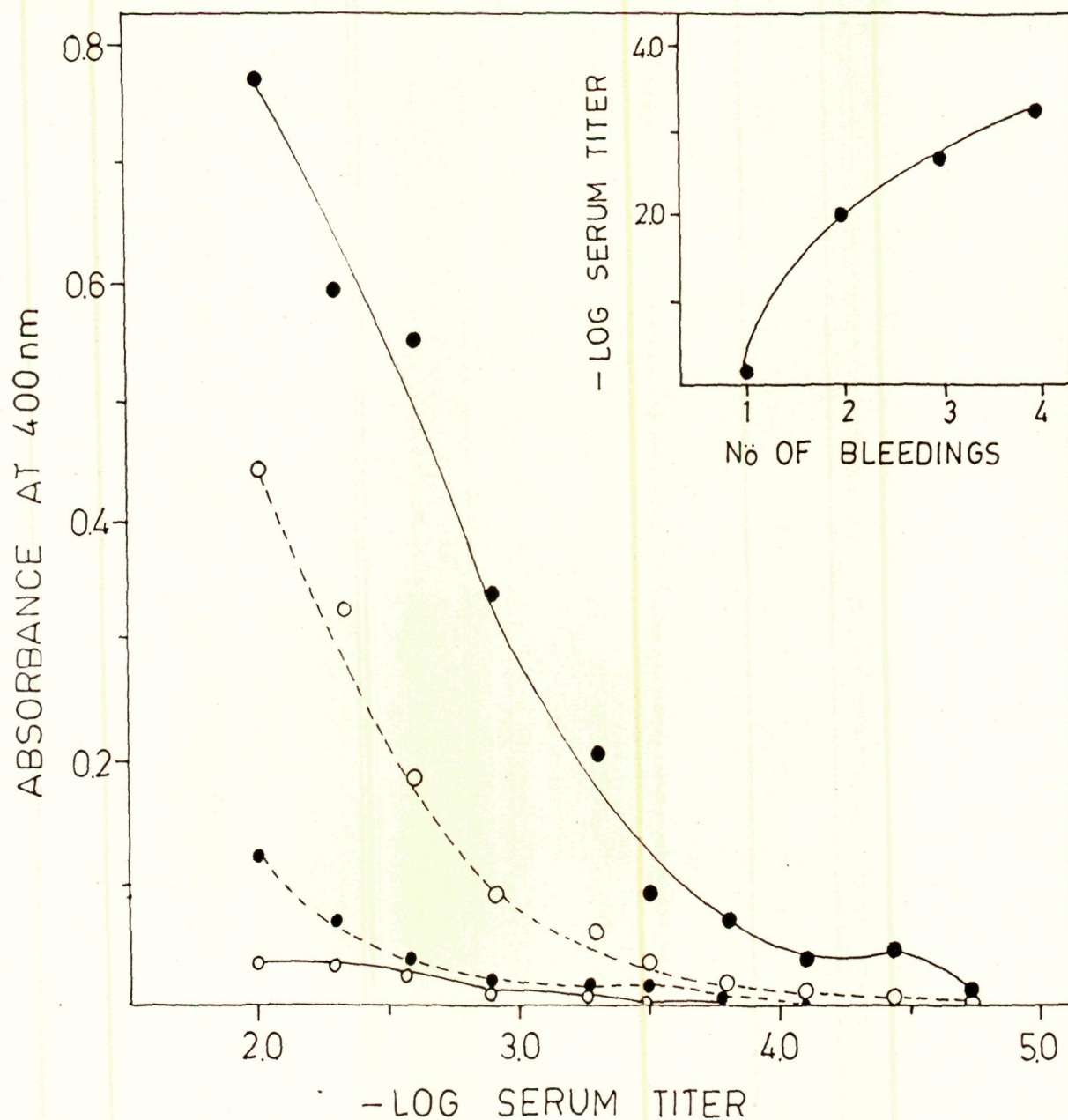
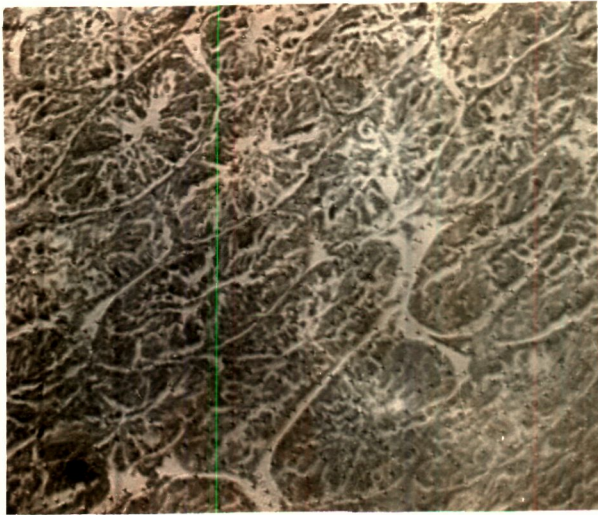


Fig.17. Direct binding of hCG by induced antibodies against irradiated hCG.

Irradiation dose: 18.2×10^7 ergs cm^{-2} . Preimmunized (—○—); immunized rabbit sera after second injection (---●---); after third injection (---○---) and after fourth injection (—●—). Inset: serum titer with respect to immunization sheduled.

A



B



81



C

Fig.18. Histology of rat testes after immunization.

A = Buffer (PBS); B = Native hCG and
C = Irradiated hCG.

of the drug was found in the bound state. The results were indicative of affinity of psoralen for the protein hormone and prompted us to a quantitative evaluation of the parameters of binding. For this purpose, we used spectroscopic methods which are rapid and require low amounts of materials for the measurements. The method was based on the quenching of intrinsic fluorescence of psoralen on binding to hCG.

(i) Ultraviolet spectra

UV absorption spectra of native and bound psoralen with hCG were taken in the spectral range of 200 nm to 370 nm. Both unbound and hCG-bound psoralen showed maximum absorption at 245 nm and 295 nm and minimum at 225 and 265 nm (Fig.19A). The hCG-psoralen complex exhibited increased absorbance over entire spectral range. When the complex (hCG-psoralen) was dialyzed extensively against phosphate buffer (0.05 M phosphate buffer, pH 7.4 containing 0.9% NaCl) the spectra showed maxima at 275 nm and a dip at 250 nm. An increased absorption as a result of complex formation was noticed but without a change in its pattern (Fig.19B).

(ii) Fluorescence spectra

Fluorescence spectra was recorded in a spectral range of 370 nm to 530 nm by exciting the samples at 330 nm, the excitation maxima of psoralen (Fig.20). There was no change in the emission wavelength of hCG-psoralen complex but a decrease in

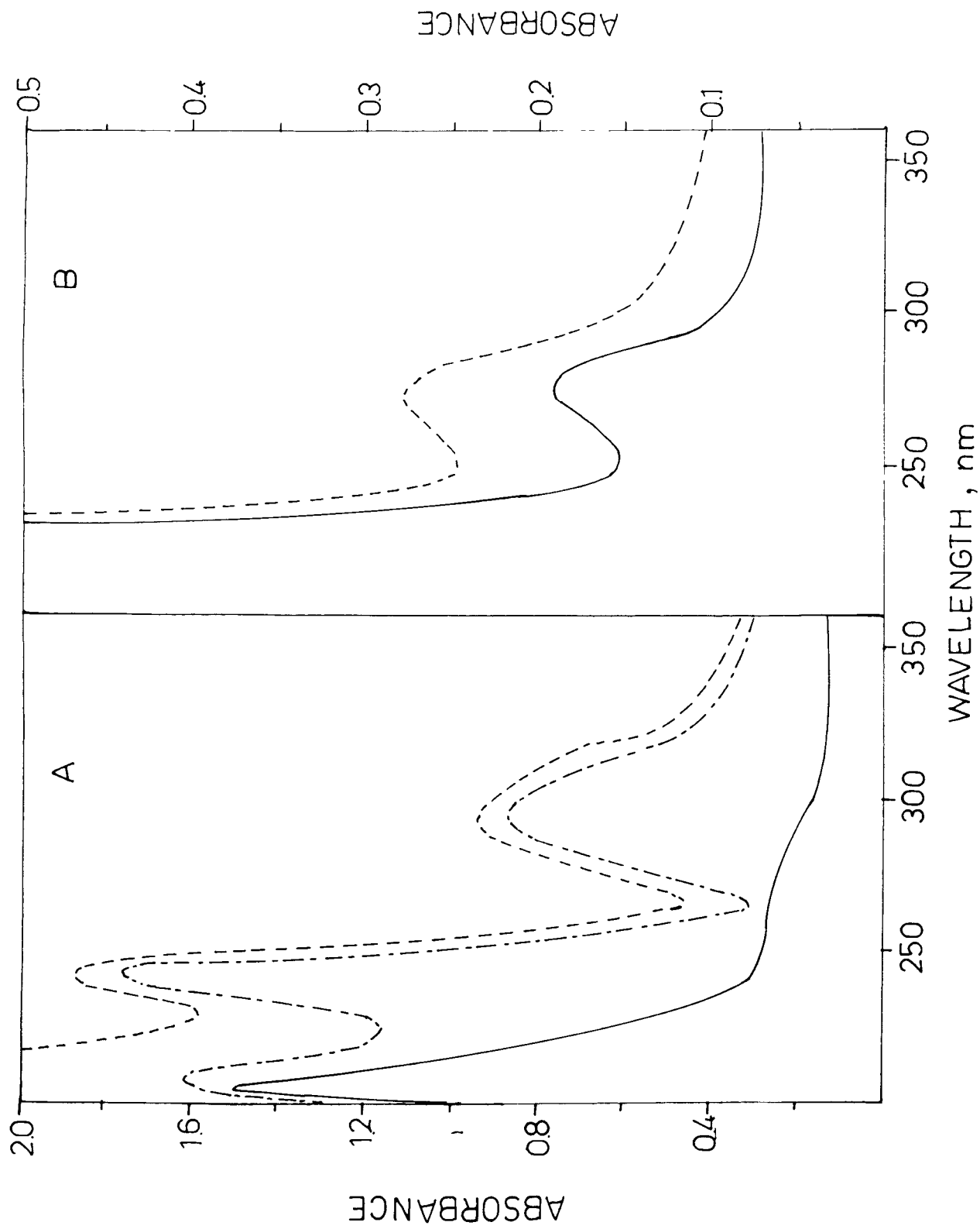


Fig.19. Ultraviolet spectra of dark interaction between psoralen and hCG.
 Hormone was in 0.05M phosphate buffer, pH 7.4 containing 0.9% NaCl.
 hCG: 1.35×10^{-6} M; psoralen: 1.07×10^{-5} M. A = before dialysis
 B = after dialysis. Native hCG (—), hCG-psoralen complex
 (-----), psoralen (-----).

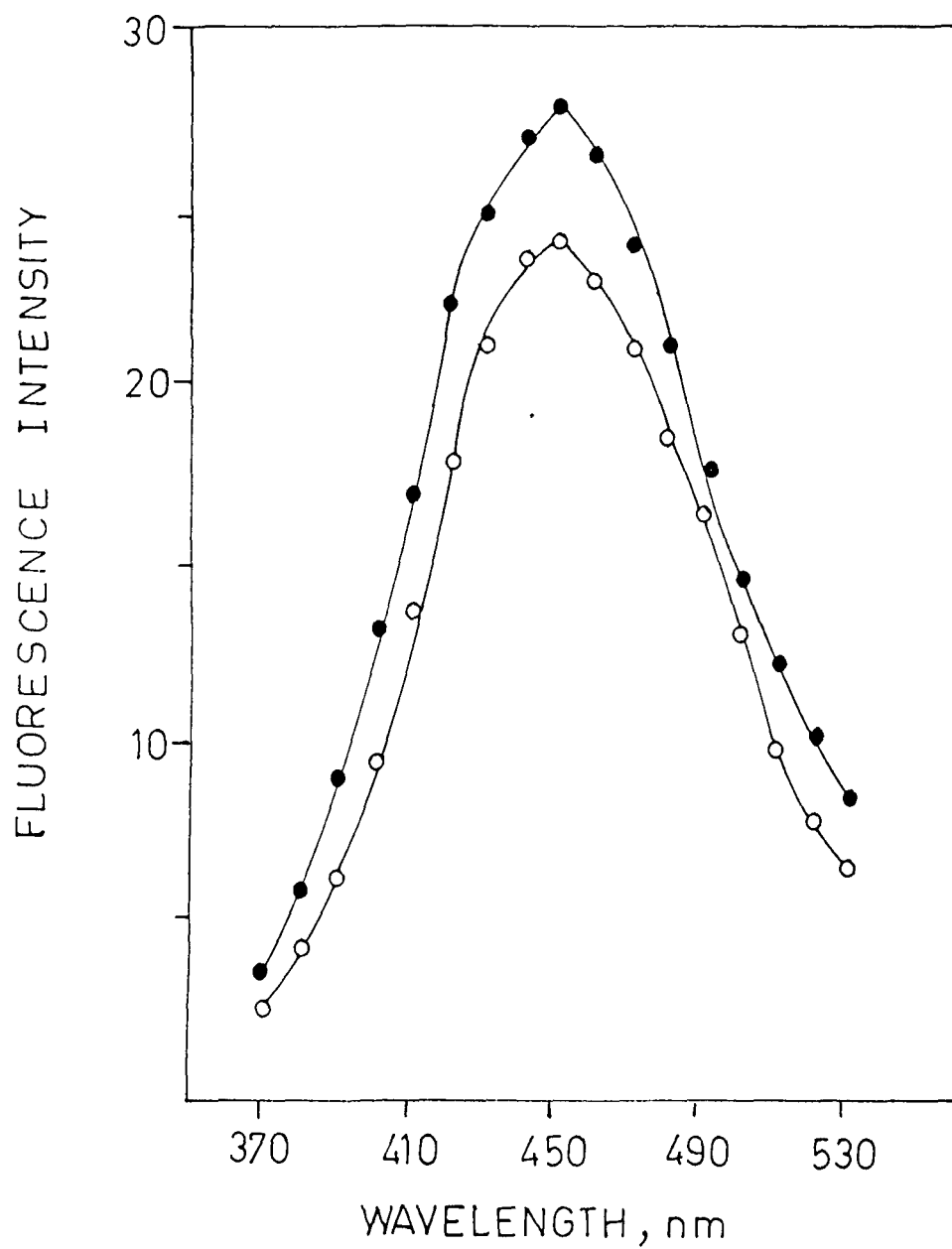


Fig.20. Fluorescence spectra of hCG-psoralen complex in dark.

Psoralen (—●—) and hCG-psoralen complex (—○—).

fluorescence intensity was noticed as compared to the fluorescence intensity of unbound psoralen.

(iii) Kinetic treatment of dark interaction

Scatchard plot for the interaction between psoralen and hCG was derived from the fluorescence quenching titration at a fixed concentration of hormone (Fig.21). The fluorescence intensity of psoralen at its emission maxima decreases upon their interaction with hCG and reaches a minimum, when the hCG gets saturated with psoralen. By assuming that the amount of fluorescence quenching is directly proportional to the amount of psoralen bound to hCG, the values of C_f and C_b were calculated, where C_f and C_b are the concentrations of free and bound psoralen respectively. The apparent association constant, K_a for interaction of hCG with psoralen was calculated by Scatchard analysis (Scatchard, 1949) which predicts that

$$\frac{r}{C_f} = K_a (n-r)$$

where, n is the apparent number of independent binding sites of hCG for psoralen, r is the ratio of concentration of bound psoralen to the total concentration of hCG used. The apparent association constant, K_a , for the drug was calculated from the slope of the Scatchard plot (Fig.21) at low r values. The plot indicated the involvement of four binding sites ($r = 3.9$) with an association constant, K_a of $0.795 \times 10^4 M^{-1}$.

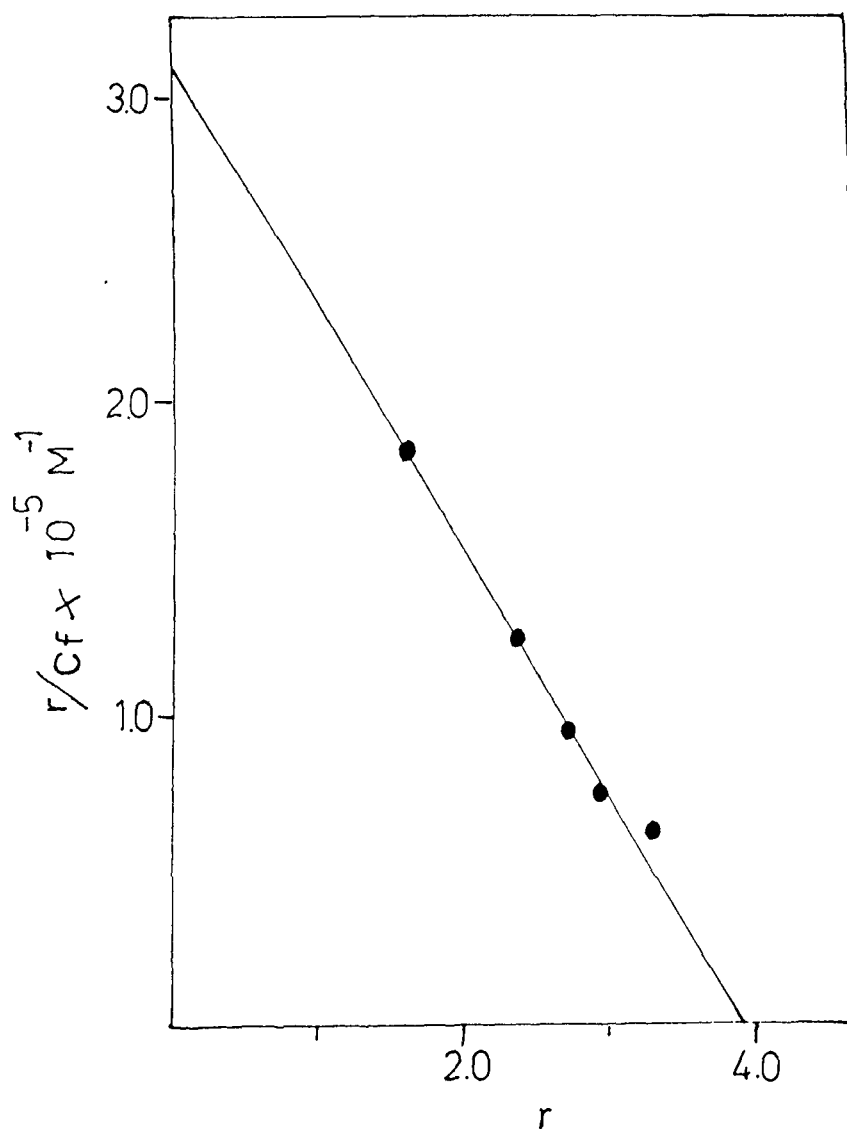


Fig.21. Scatchard plot for binding of psoralen to hCG in dark.

The concentration of hCG was kept constant ($1.35 \times 10^{-6} \text{ M}$) while that of psoralen was varied from 1.07×10^{-5} to $5.38 \times 10^{-5} \text{ M}$. Fluorescence emission intensity was measured at the excitation maxima of psoralen.

In Fig.22 the binding parameter r vs C_f has been computed. With increase in free psoralen concentration, the number of binding sites have also increased. Under these experimental conditions, the percent quenching of psoralen fluorescence is decreased (Fig.23).

(iv) Gel filtration

In addition to fluorescence quenching, the binding capacity of psoralen to hCG was also checked by gel filtration experiments. There was no change in elution volume of hCG and hCG-psoralen complex but a significant increase in 280 nm absorption of the complex was noted (Fig.24).

11. Inhibition by Psoralen in Immunoreactivity of hCG-Anti hCG Interaction

Psoralen caused a concentration dependent inhibition of hCG-anti-hCG-antibody interaction as depicted in Fig.25. With a increase in the concentration of psoralen, there is gradual decrease in immunoreactivity. A parallelism between two parameters was obtained at a saturation point (i.e. 60 ng of psoralen) followed by a plateau which is persistent till 100 ng of psoralen.

12. Detection of Antibodies in Immune Sera

Antibodies raised in rabbits against hCG and hCG psoralen complex with Freund's complete adjuvant were checked for

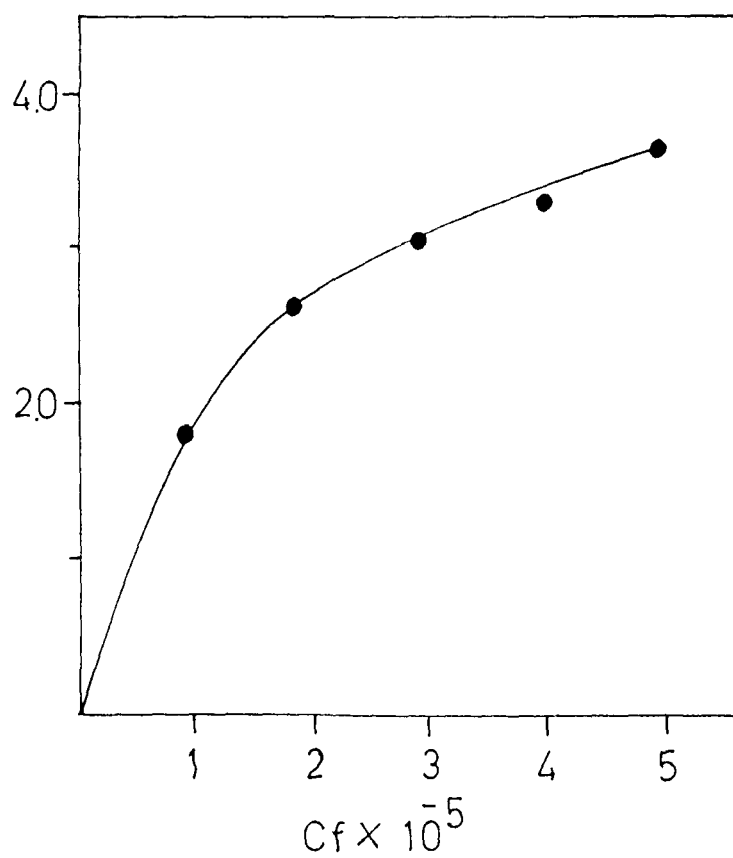


Fig.22. Plot of binding parameters of hCG-psoralen complex.

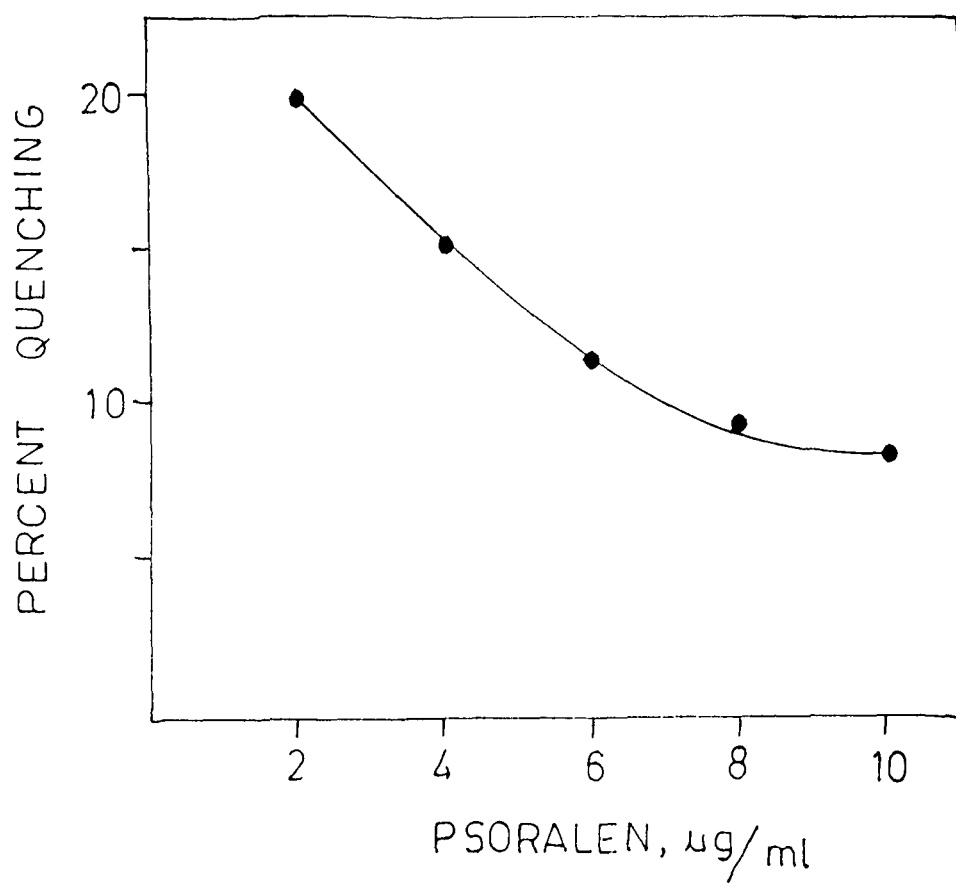


Fig.23. Plot of binding of psoralen to hCG in terms of percent quenching.

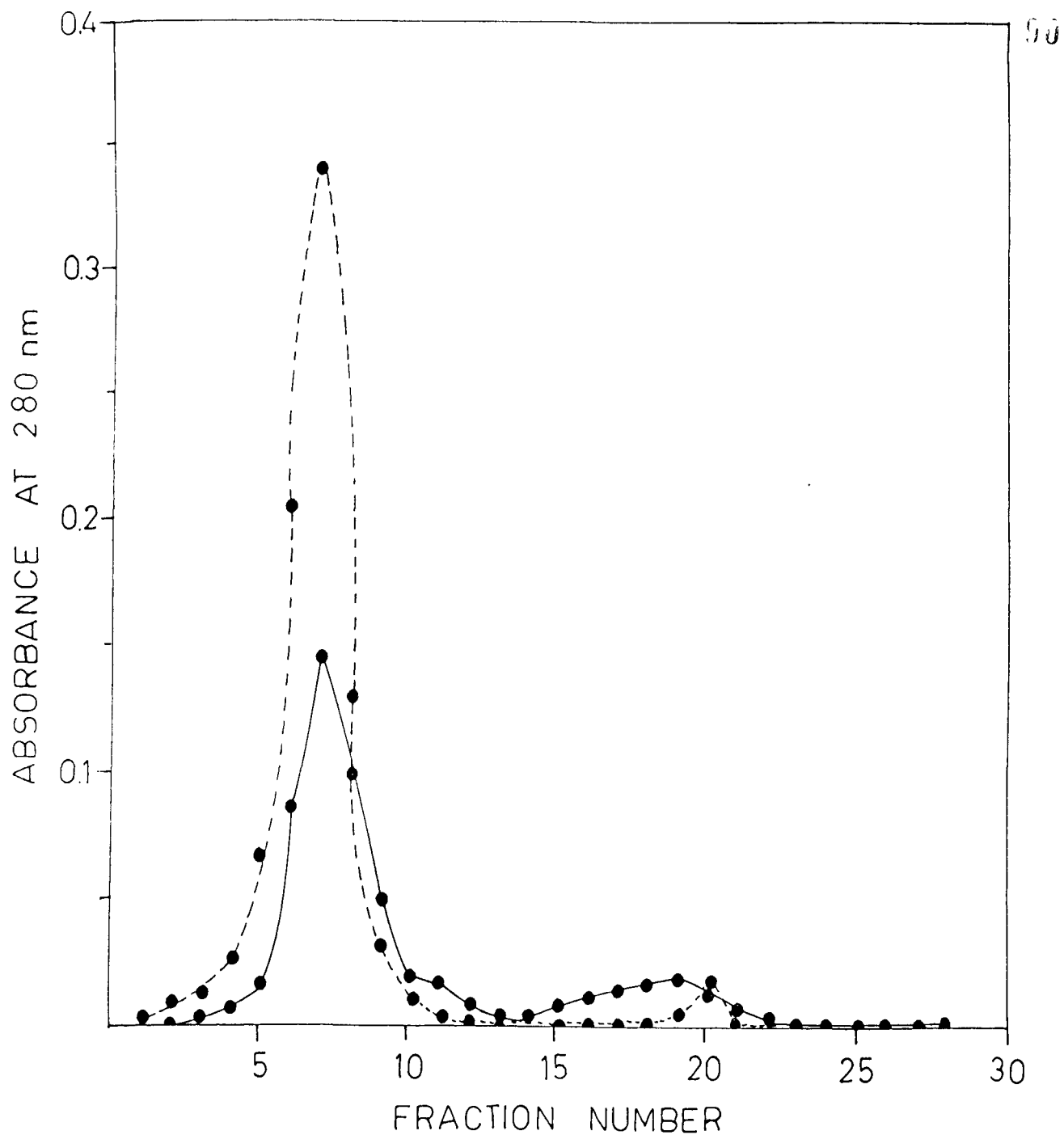


Fig.24. Hydrodynamic behaviour of hCG and hCG-psoralen complex on Sephadex G-100 column.

hCG (—●—) and hCG psoralen complex
(- - -●- - -).

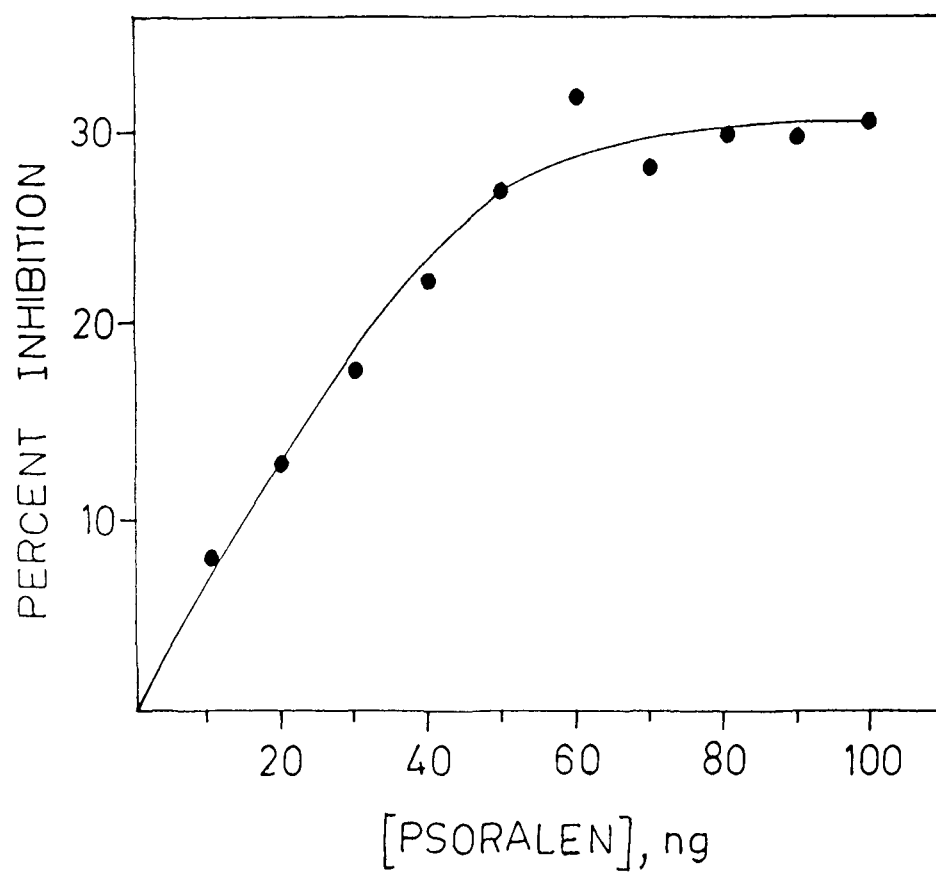


Fig.25. Effect of psoralen on hCG-anti-hCG antibody binding capacity.

reactivity by ELISA on polystyrene plates.(Fig.26). After third injection, the level of antibodies was progressively increased. The titers of antisera were 1:1920, 1:15360 and 1:491520 after third, fourth and fifth injections respectively. No appreciable antibody activity was found with preimmunized rabbit serum. The complex was found to be more antigenic than unbound hCG (Fig.27).

IgG isolated from hCG psoralen antisera was checked for binding characteristics. Fig.28 shows the results obtained with different concentration of IgG in ELISA using hCG and hCG-psoralen complex as antigens. The complex was found to be more reactive than unbound hCG for anti-hCG-psoralen IgG.

13. Specificity of Anti-hCG Antibodies

The specificity of anti-hCG antibodies was checked by inhibition experiments. Varying concentrations of inhibitors (hCG, β hCG and α hCG) were mixed with 1:5000 diluted serum and incubated at 37°C for 2 hrs and at 4°C for 18 hrs.

Maximum inhibition was obtained by hCG followed by alpha and beta subunits (Fig.29). The respective values were 60%, 55% and 33%.

14. Specificity of Anti-hCG-Psoralen Antibodies

(1) With antisera

Fig.30 shows the results obtained by inhibition ELISA. High degree of specificity was evident since 68% inhibition was

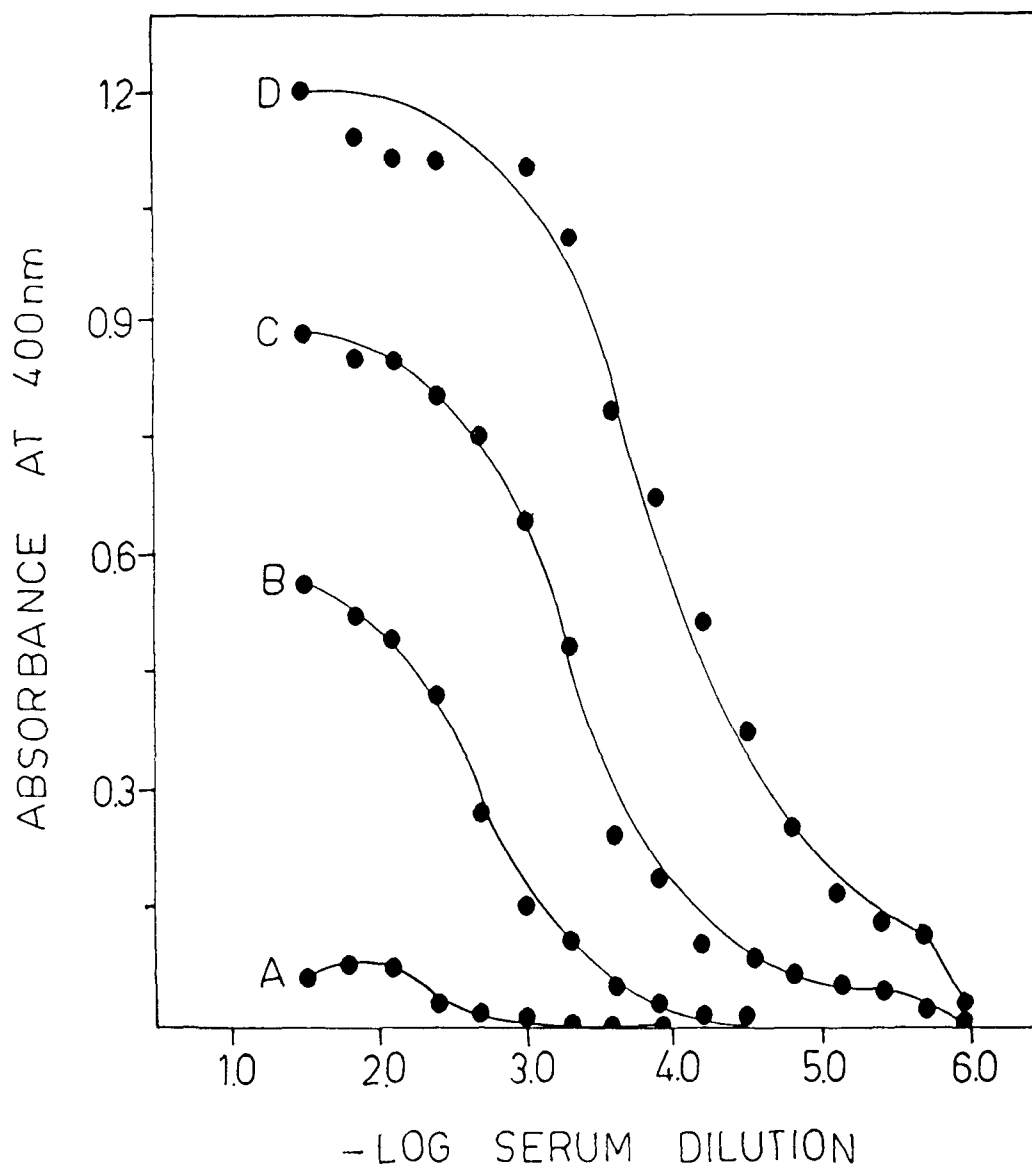


Fig.26. Binding of hCG by induced antibodies against hCG-psoralen complex.

A = preimmunized sera; B, C and D are hCG-psoralen immunized sera after third, fourth and fifth injections respectively.

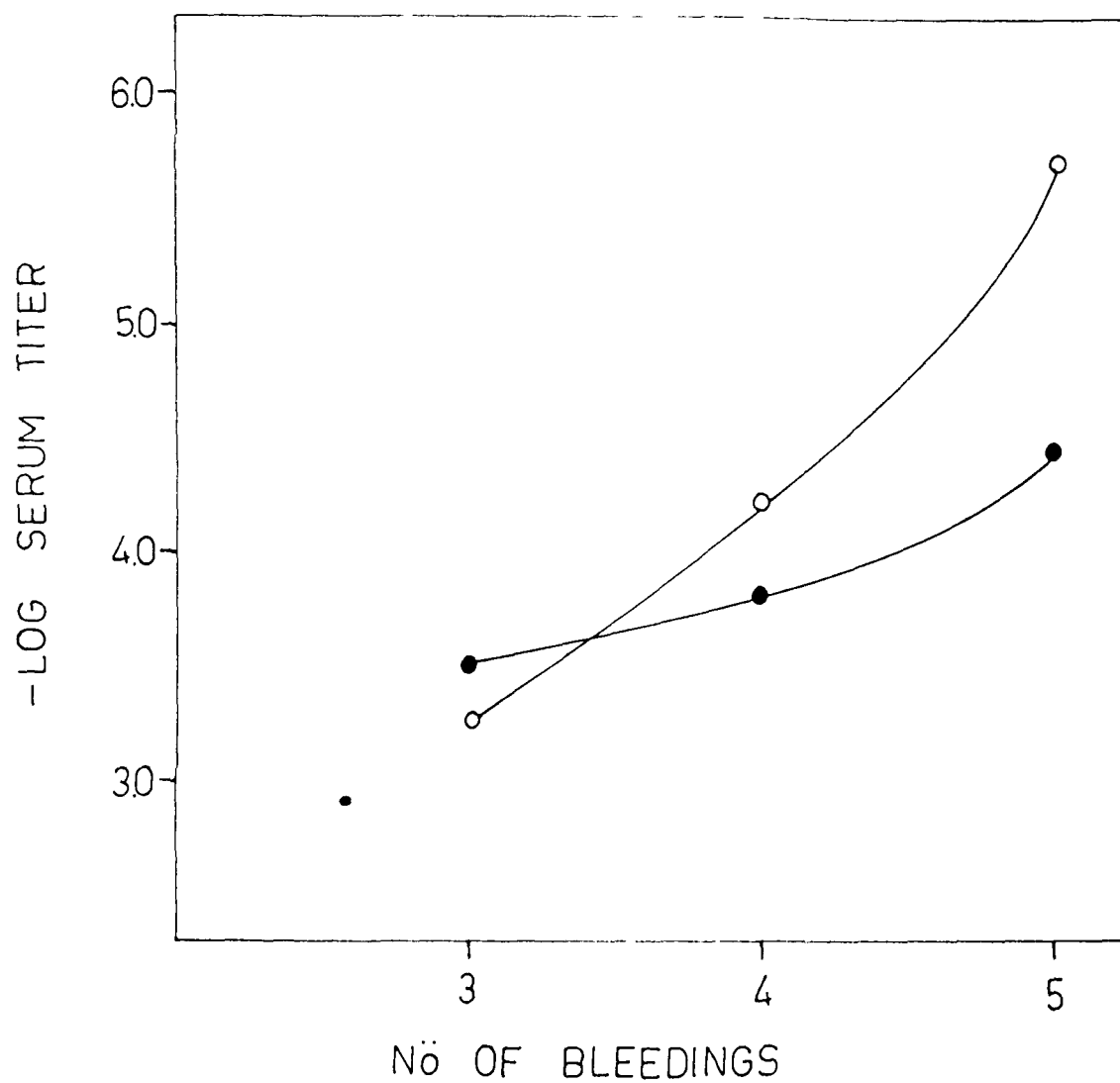


Fig.27. A comparative study of titers of induced antibodies against hCG and hCG-psoralen complex.

hCG (—●—) and hCG-psoralen complex
(—○—).

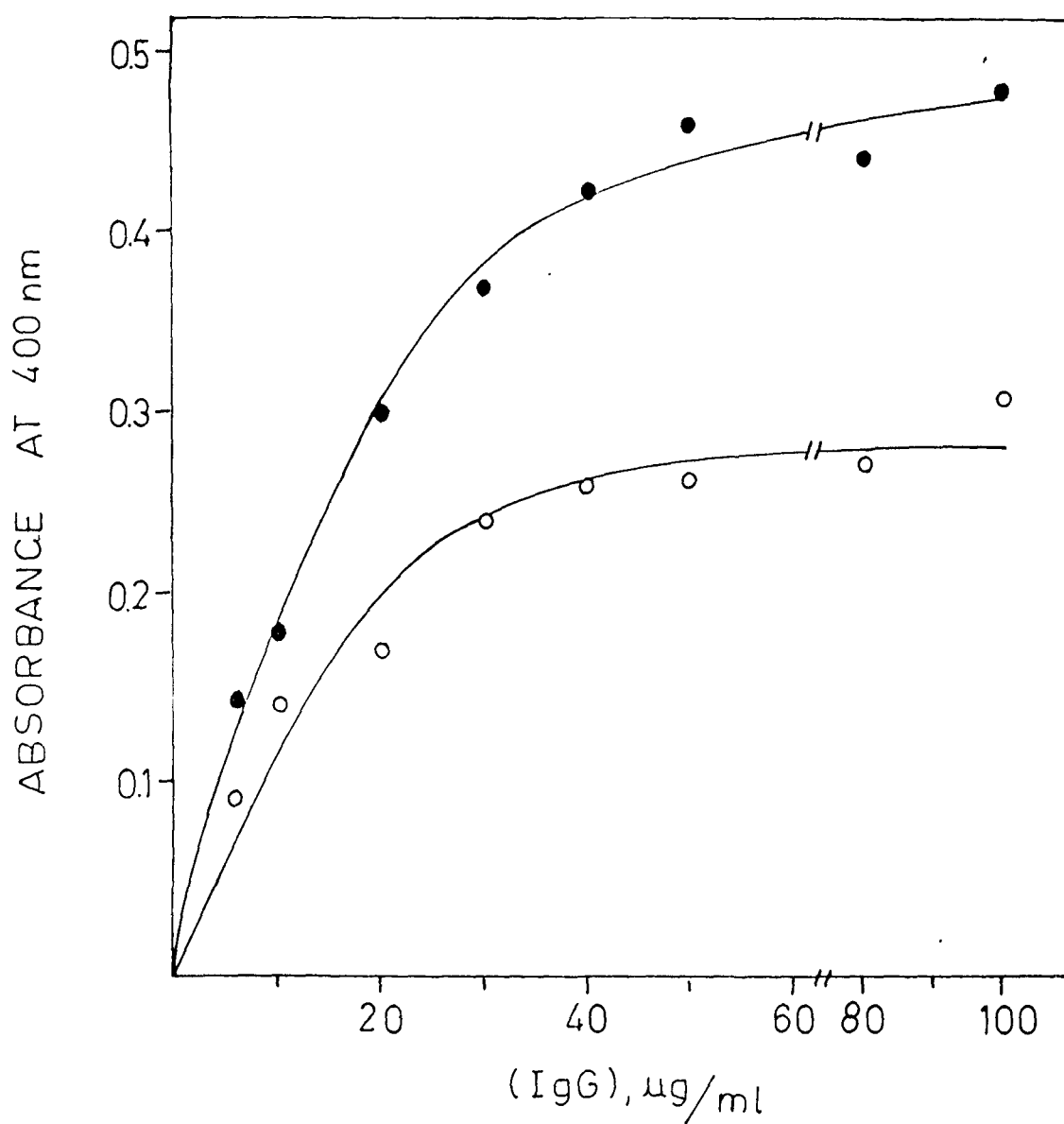


Fig.28. Antibody activity of anti-hCG-psoralen IgG.

hCG (—○—) and hCG-psoralen (—●—)
were used as antigens.

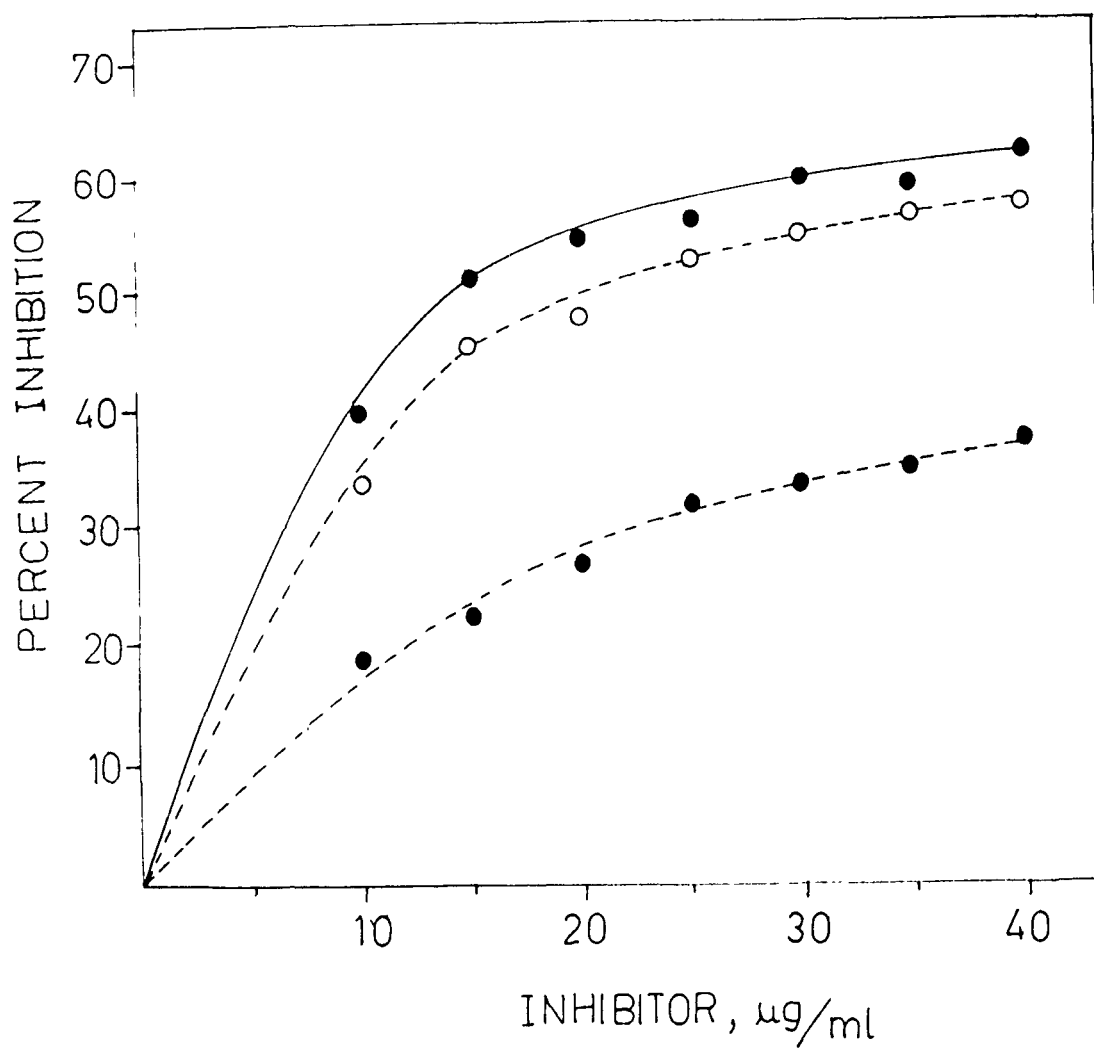


Fig.29. Inhibition ELISA with anti-hCG antibodies.

Inhibitors were hCG (—●—), alpha-subunit (---○---) and beta-subunit (---●---).

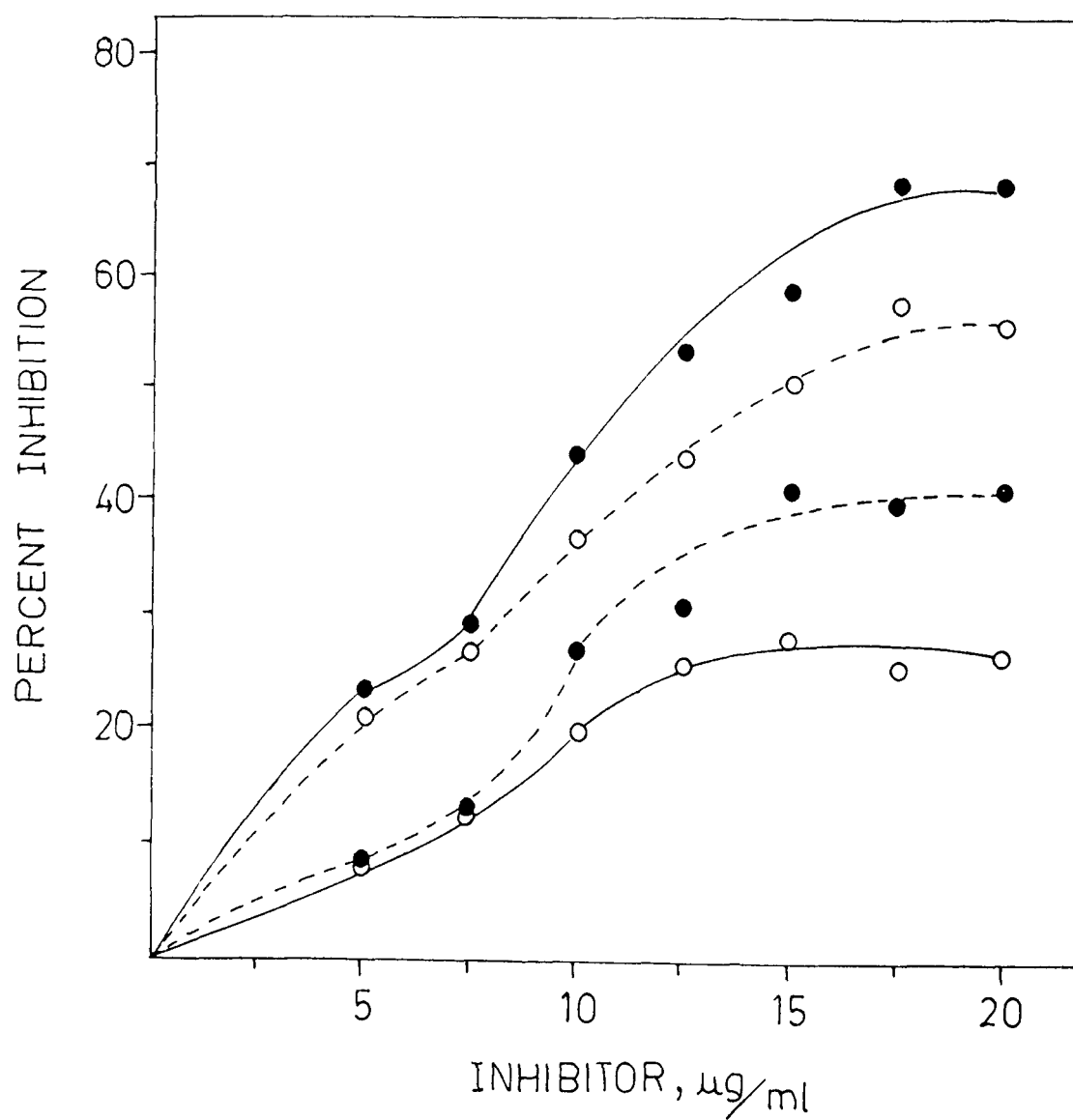


Fig.30. Inhibition ELISA with anti-hCG-psoralen antibodies.

Inhibitors were hCG (---○---), hCG-psoralen complex (—●—), alpha-subunit (---●---) and beta-subunit (—○—).

achieved with hCG-psoralen followed by 56% with hCG, 42% with alpha subunit and 26.31% with beta subunit of hCG at an inhibitor concentration of 20 $\mu\text{g/ml}$.

(11) With isolated IgG

Varying concentrations of inhibitors (hCG-psoralen, hCG, alpha subunit and beta subunit, 0.1 μg - 2.5 μg) were mixed with 5 μg of anticomplex IgG. The extent of inhibition obtained with hCG-psoralen, hCG, alpha subunit and beta subunit was 54%, 48%, 30% and 20.3% respectively at an inhibitor concentration of 1.0 μg (Fig.31).

15. Effect of UV-A Light upon Binding of Psoralen to hCG

(1) Ultraviolet measurements

HCG, psoralen and hCG-psoralen complex were irradiated with UV-A (365 nm) light at a dose rate $10.2 \times 10^8 \text{ ergs cm}^{-2} \text{ min}^{-1}$. UV spectra after irradiation was taken in a spectral range of 230 nm to 400 nm before and after extensive dialysis against phosphate buffer (Fig.32). Both hCG and hCG-psoralen complex after irradiation showed similar pattern of spectra but the complex exhibited increased absorbance over entire spectral range. The difference spectra (Fig.32C) is indicative of psoralen interaction with hCG on exposure to UV-A light.

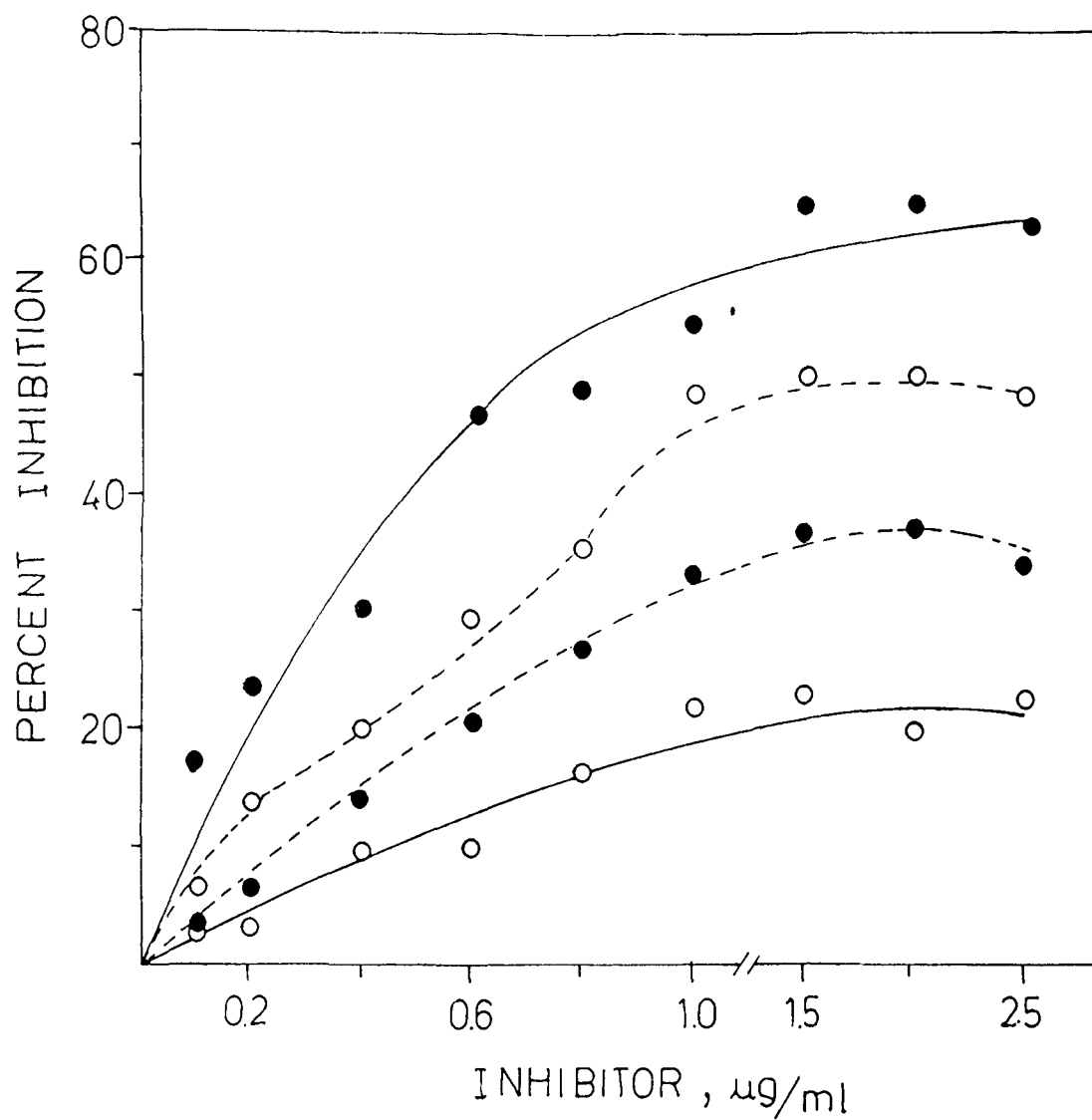


Fig.31. Inhibition ELISA with anti-hCG-psoralen IgG.

Inhibitors were hCG-psoralen complex (—●—),
hCG (---○---), alpha-subunit (---●---) and beta-
subunit (—○—).

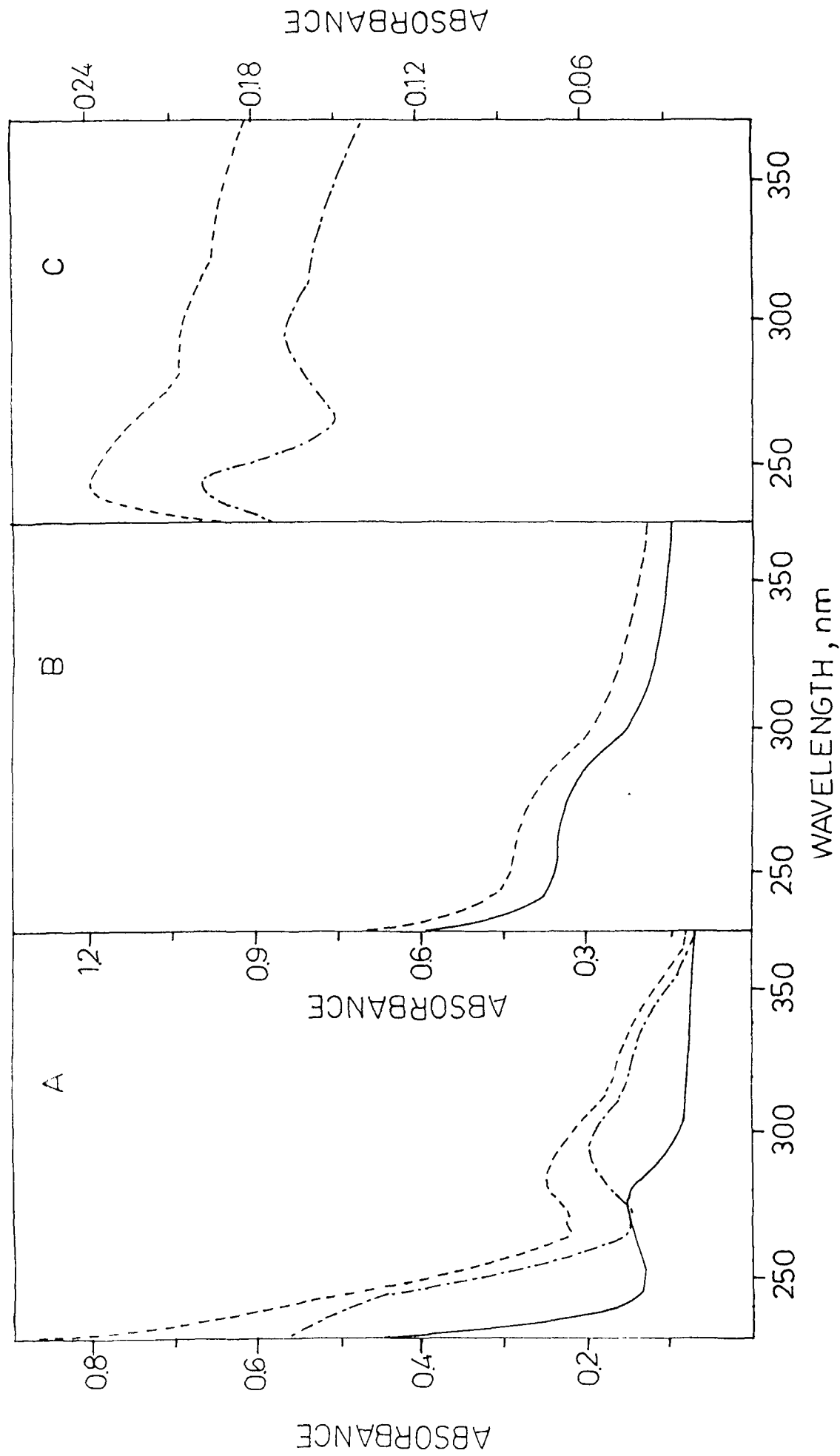


Fig.32. Ultraviolet spectra of UV-A irradiated hCG-psoralen complex.

A = before dialysis; B = after dialysis; C = difference spectra. hCG (—); psoralen (- - - - -) and hCG-psoralen complex (······).

(ii) Fluorescence measurements

A fixed concentration of hCG ($1.35 \times 10^{-6} \text{M}$) and psoralen ($1.07 \times 10^{-5} \text{M}$) were UV-A irradiated for different time periods (Fig.33). As the radiation time was increased, fluorescence intensity of the complex and psoralen was increased and there was a linear relationship between the difference in fluorescence intensity of psoralen and hCG-psoralen complex (Fig.33 inset).

(iii) Effect of drug concentration upon binding

A fixed concentration of hCG ($1.35 \times 10^{-6} \text{M}$) and a varying concentration of psoralen ($1.07 \times 10^{-5} \text{M}$ to $5.38 \times 10^{-5} \text{M}$) were mixed and irradiated as described in Experimental. It was found that with increase in drug concentration the fluorescence intensity was also increased (Fig.34).

16. Fluorescence Transition of hCG in 8M Urea

Kinetics of unfolding of hCG was followed by monitoring loss in fluorescence intensity with respect to denaturation time. The fluorescence transition curve for hCG was constructed by plotting fractions denatured, f_D vs time (Fig.35). The hormone was mixed with urea in a final concentration of $200 \mu\text{g/ml}$ in 8 M urea and fluorescence intensity was monitored at 350 nm. The kinetic analysis of the denaturation process was performed by the method of Warren et al (1974) using first order rate equation:

$$-\log (Y_{\infty} - Y_t) = mD + C$$

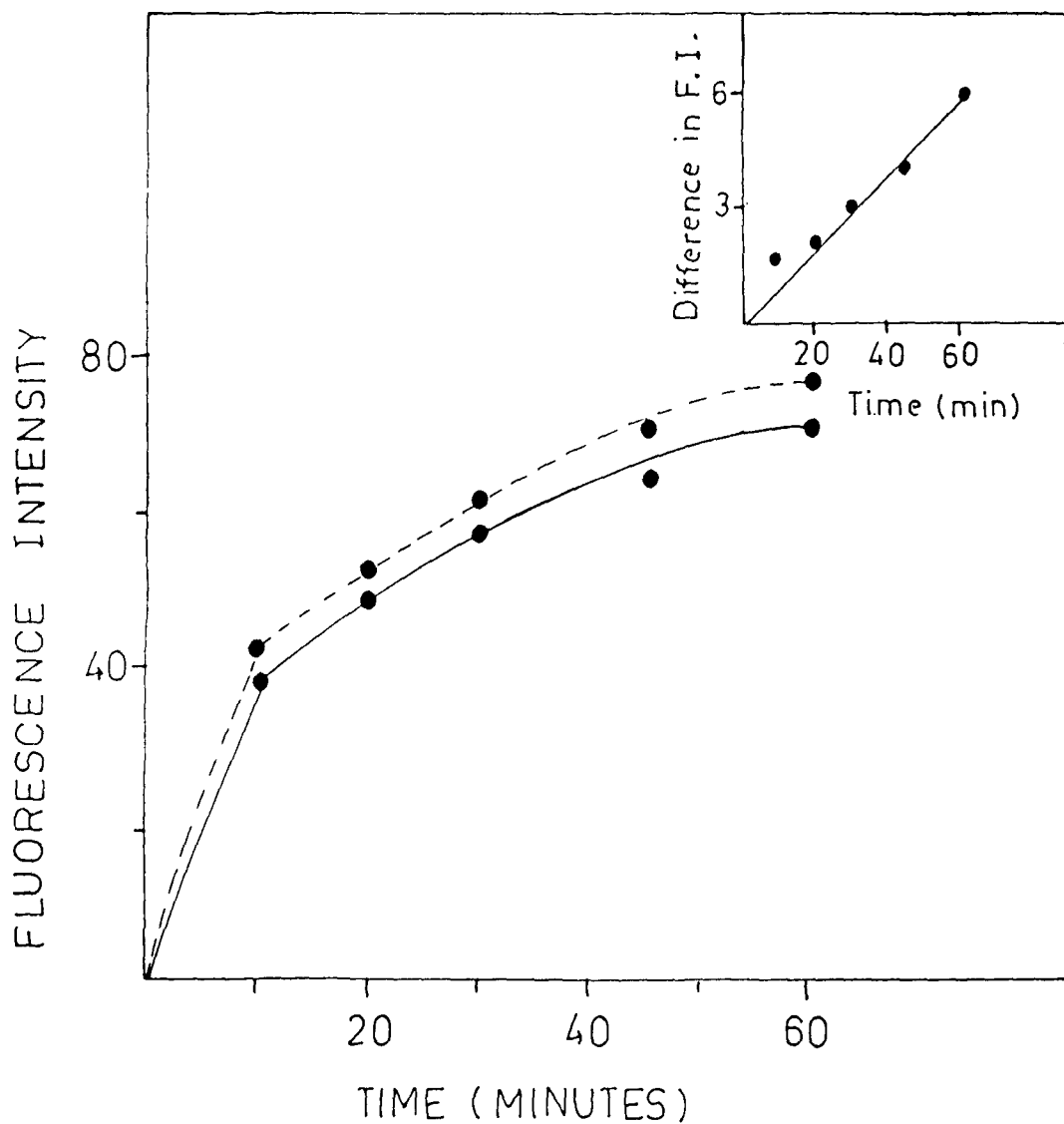


Fig.33. Effect of irradiation time on binding capacity of psoralen to hCG.

hCG-psoralen complex (---●---) and psoralen (—●—). Inset: Difference in fluorescence intensities of hCG-psoralen complex and psoralen with respect to time.

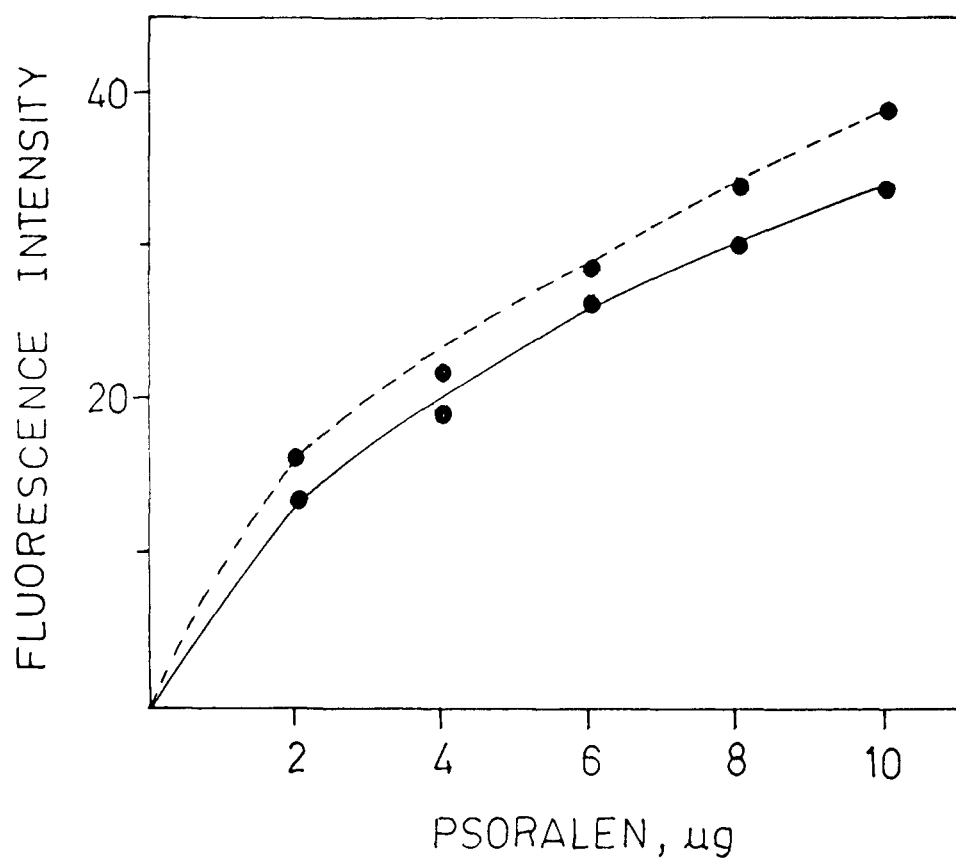


Fig.34. Effect of psoralen concentration upon binding to hCG in presence of UV-A light.

hCG-psoralen complex (--●--) and psoralen (—●—).

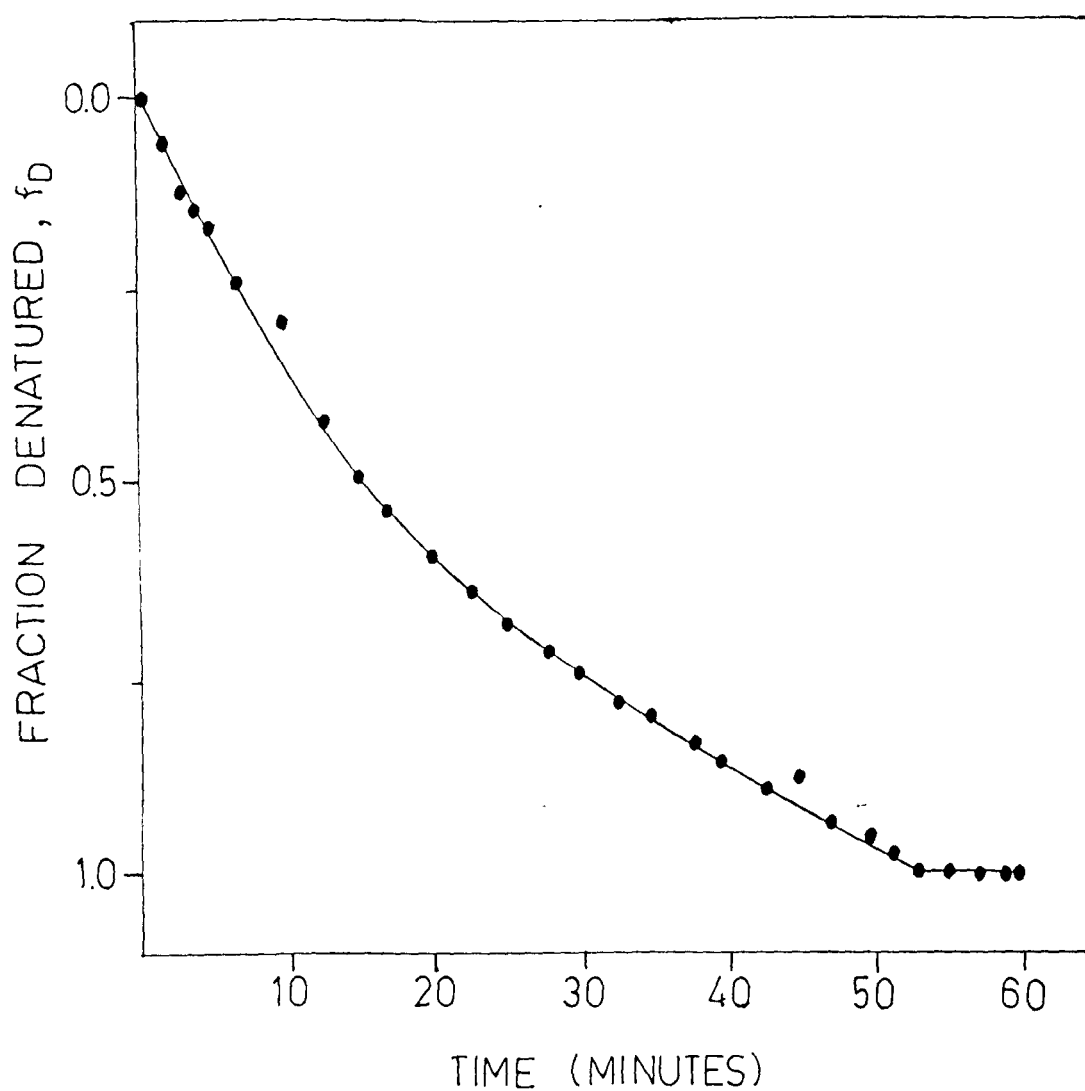


Fig.35. Effect of urea on stability of hCG.

Values of f_D were calculated using equation given in experimental. hCG concentration $1.35 \times 10^{-6} \text{ M}$ in 0.05M phosphate, pH 7.4 containing 0.9% of NaCl; Urea: 8M.

where Y_{∞} and Y_t are the percent change in fluorescence intensity of hCG at the end of denaturation process (1 hr, Y_{∞}) and at particular time respectively (Y_t). The $-\log (Y_{\infty} - Y_t)$ values were plotted against denaturation time and slope of the line, m , was related to the apparent first order rate constant for denaturation process. Plot of fluorescence transmission (Fig.35) showed the process to occur in a single phase.

17. Dark Binding of Psoralen to Subunits of hCG

In order to study the binding of psoralen to alpha and beta subunits of hCG, the fluorometric properties of free and bound (with subunits) psoralen were compared. The alpha-psoralen and beta-psoralen complexes exhibited no change in relative excitation and emission wavelenths but a significant quenching was observed (Fig.36 and 37).

(1) Ultraviolet spectra

The UV spectra of psoralen and its complex with alpha and beta subunits was recorded (Fig.38). A slight shift in wavelength of the alpha-psoralen complex was noted. Psoralen exhibited two characteristic maxima one at 245 nm and other at 295 nm, The alpha-psoralen complex showed a single emission peak at 290 nm (Fig.38). Similarly beta-psoralen complex have a maxima at 285 nm, different from psoralen maxima of 295 nm (Fig.39).

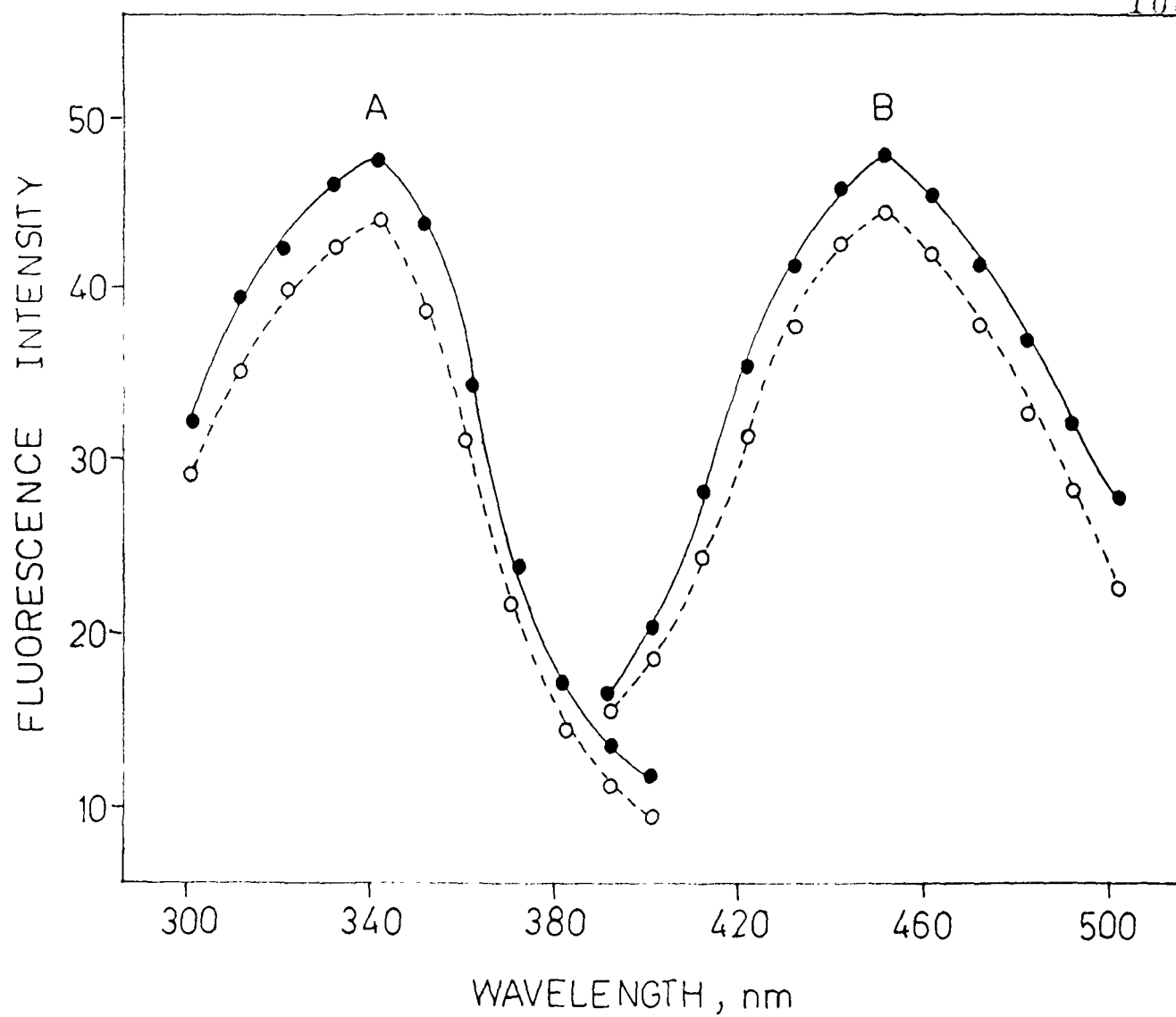


Fig.36. Fluorescence spectra of dark interaction between alpha-subunit and psoralen.

A = excitation spectra; B = emission spectra.

Psoralen (—●—) and alpha-psoralen complex (--○--)

Alpha-subunit: $6.6 \times 10^{-5} \text{M}$ and psoralen: $1.07 \times 10^{-4} \text{M}$.

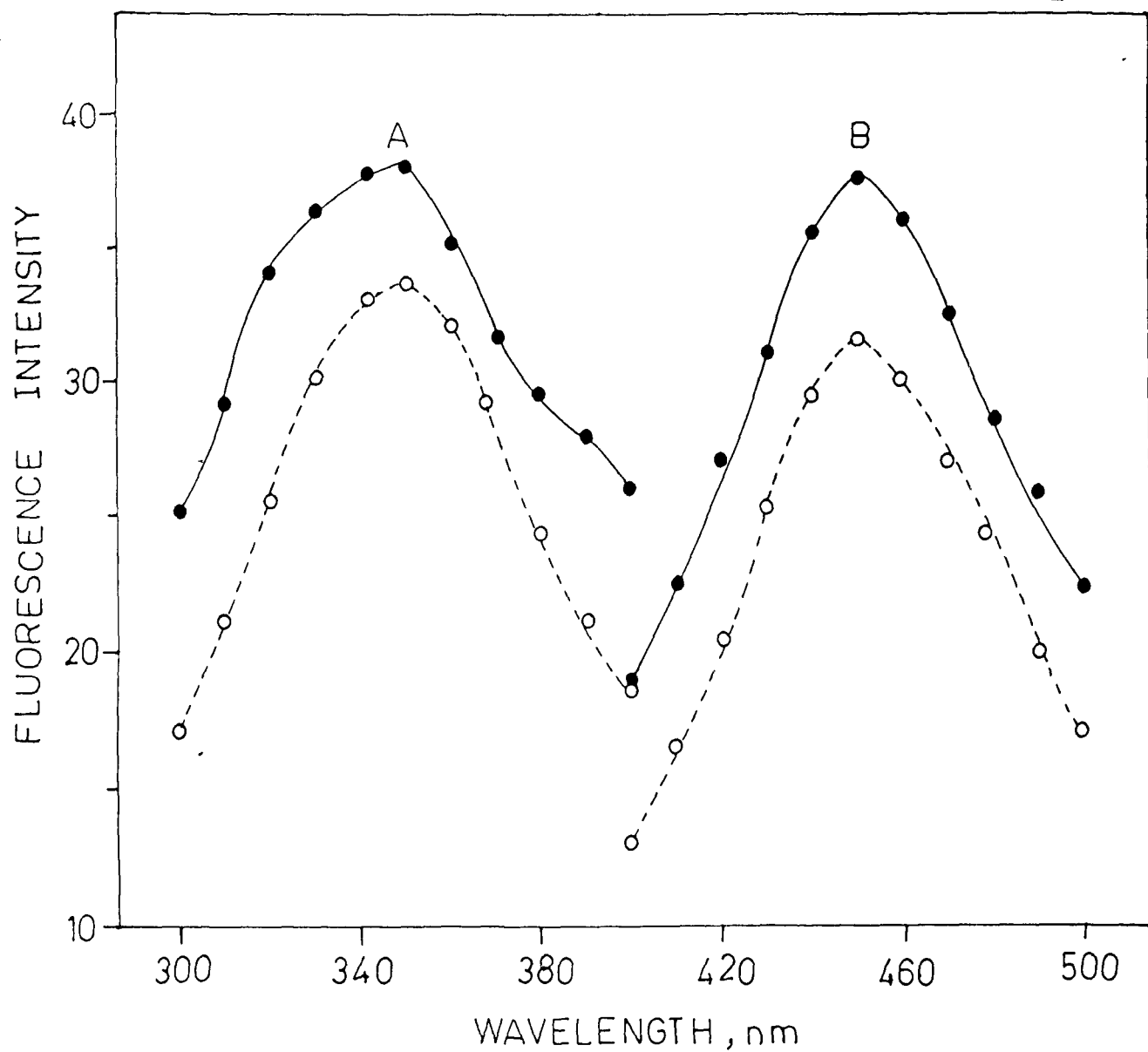


Fig.37. Fluorescence spectra of dark interaction between beta-subunit and psoralen.

A = excitation spectra; B = emission spectra.

Psoralen (—●—) and beta-subunit (--○--).

Beta-subunit: 4.3×10^{-5} M and psoralen: 1.07×10^{-4} M.

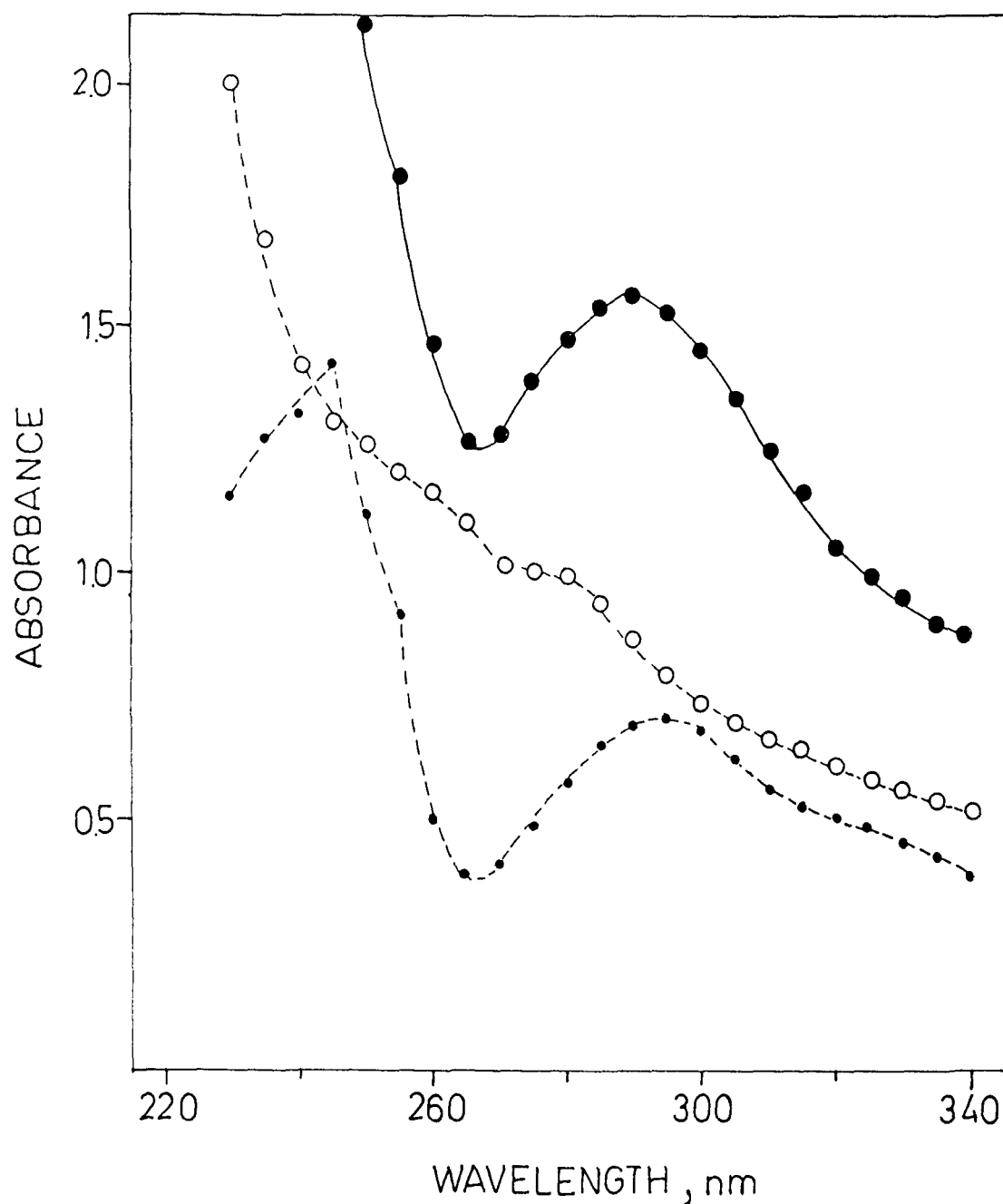


Fig.38. Ultraviolet spectra of psoralen bound alpha-subunit in dark.

Alpha-subunit (----○----); psoralen (----●----) and alpha-psoralen complex (—●—).

Alpha-subunit: $6.6 \times 10^{-5} \text{M}$; psoralen = $1.07 \times 10^{-4} \text{M}$.

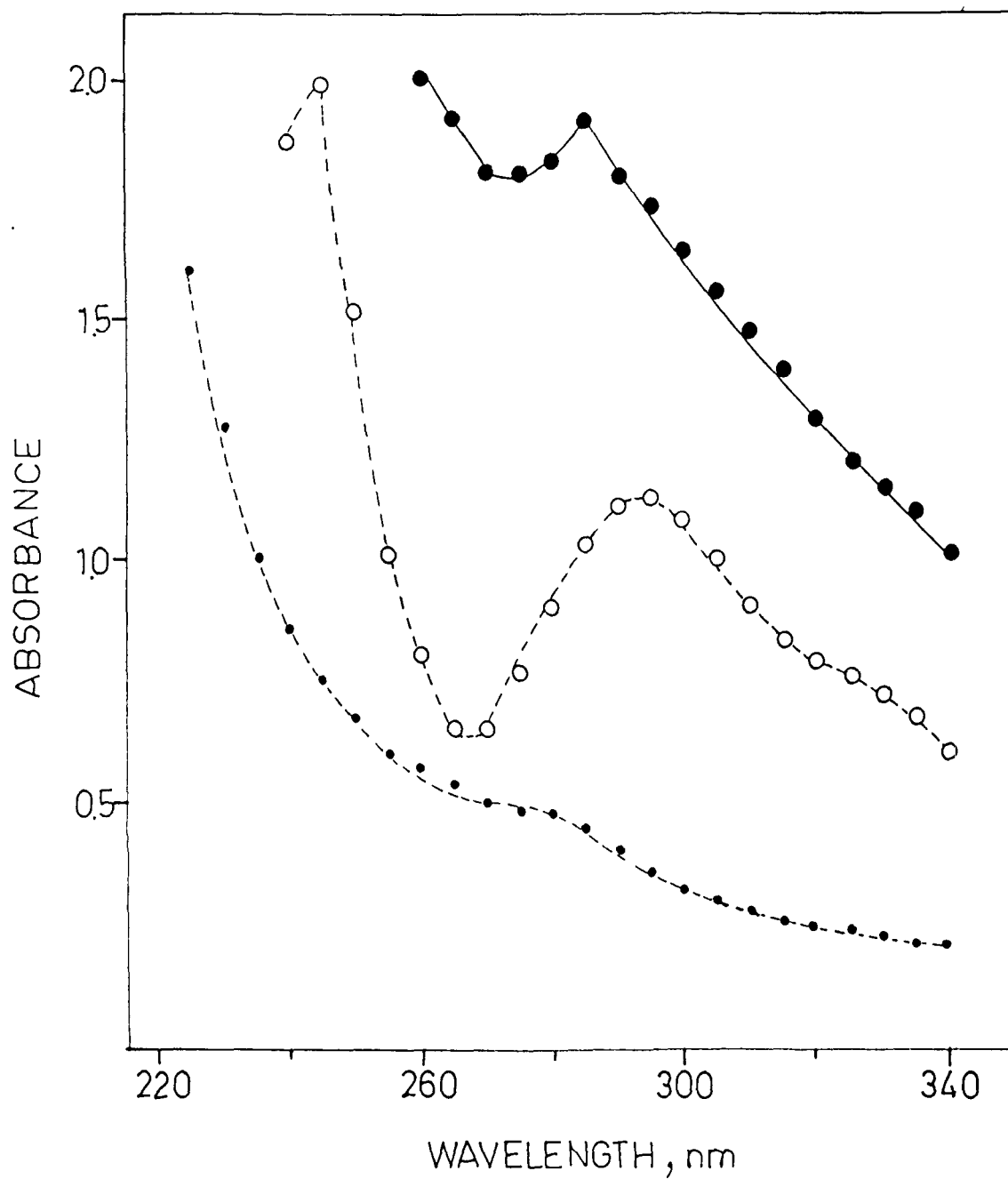


Fig.39. Ultraviolet spectra of psoralen bound beta-subunit in dark.

Beta-subunit (---•---); psoralen (---○---)
and beta-psoralen complex (—●—).

Beta-subunit: $4.3 \times 10^{-5} \text{ M}$; psoralen: $5.38 \times 10^{-4} \text{ M}$.

(11) Gel filtration

In addition to spectroscopic studies the binding of psoralen to subunits was checked by gel filtration experiments. There was no change in elution behaviour of alpha subunit and alpha-psoralen complex but a increased absorbance was noticed (Fig.40A). Similar results were obtained with beta subunit and beta-psoralen complex (Fig.40B).

18. Antigenicity of Subunits

Antibodies against alpha-subunit, alpha-psoralen, beta-subunit and beta-psoralen complex were raised in rabbit with Freund's complete adjuvant and their titer was determined by ELISA. The direct binding of anti-alpha subunit and anti-alpha-psoralen complex antibodies with hCG-showed a titer of 1:51200 and 1:3200 respectively (Fig.41). Similarly, the titer of anti-beta subunit and anti-beta-psoralen complex antibodies was 1:51200 and 1:1600 respectively (Fig.42). Normal rabbit sera did not show any appreciable binding.

The antigenicity of subunits and their complexes were evaluated after each immunization. Subunits of hCG appears to be more antigenic than their respective complexes (Fig. 43 and 44).

19. Chymotrypsin Digestion of Beta-Subunit

As a result of digestion by α -chymotrypsin of β -subunit, three peptides were obtained by gel filtration on Sephadex G-25.

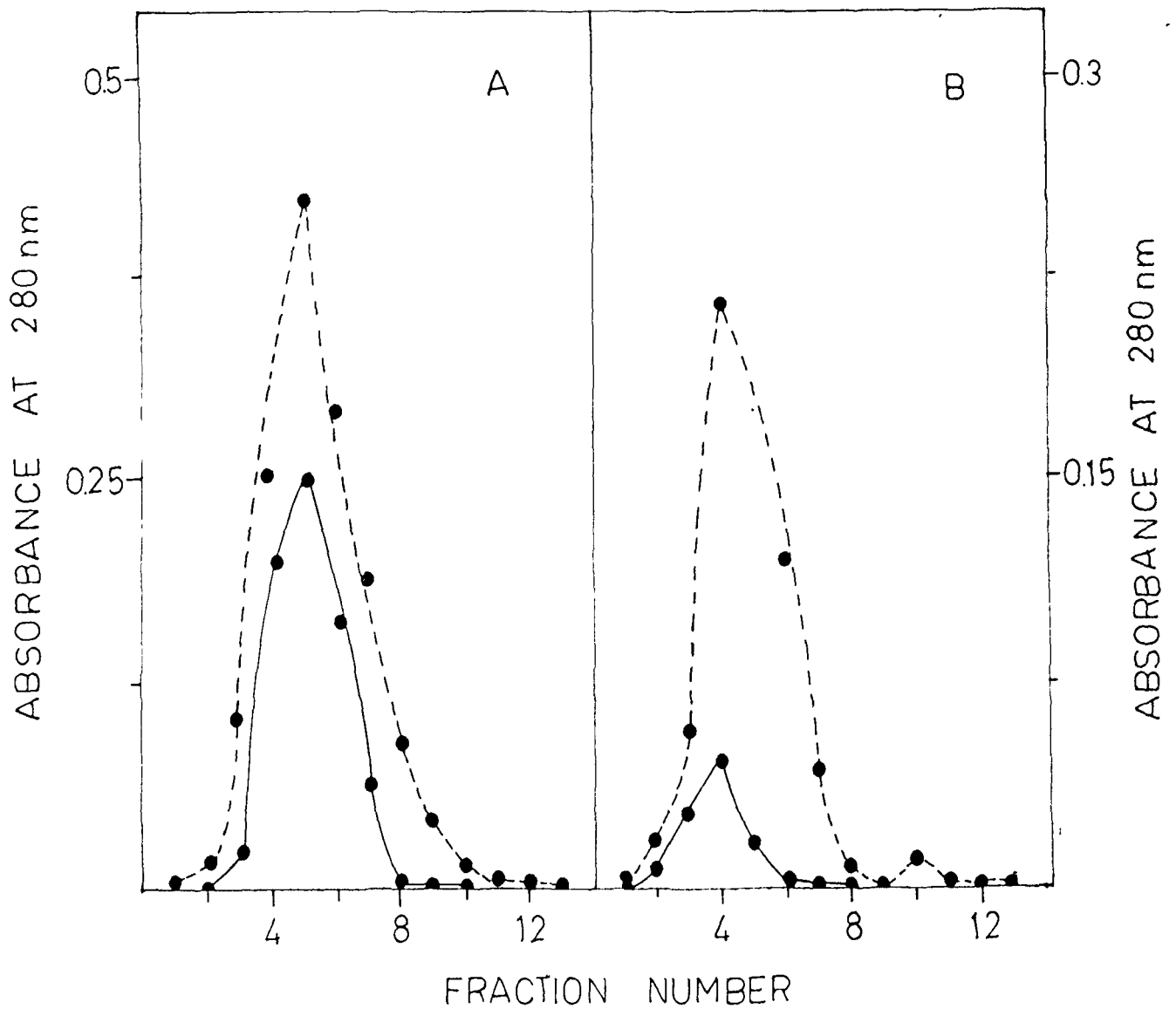


Fig.40. Hydrodynamic behaviour of subunits of hCG and its complex with psoralen on Sephadex G-100 column.

A = alpha-subunit (—●—) and alpha-psoralen complex (---●---).

B = beta-subunit (—●—) and beta-psoralen complex (---●---).

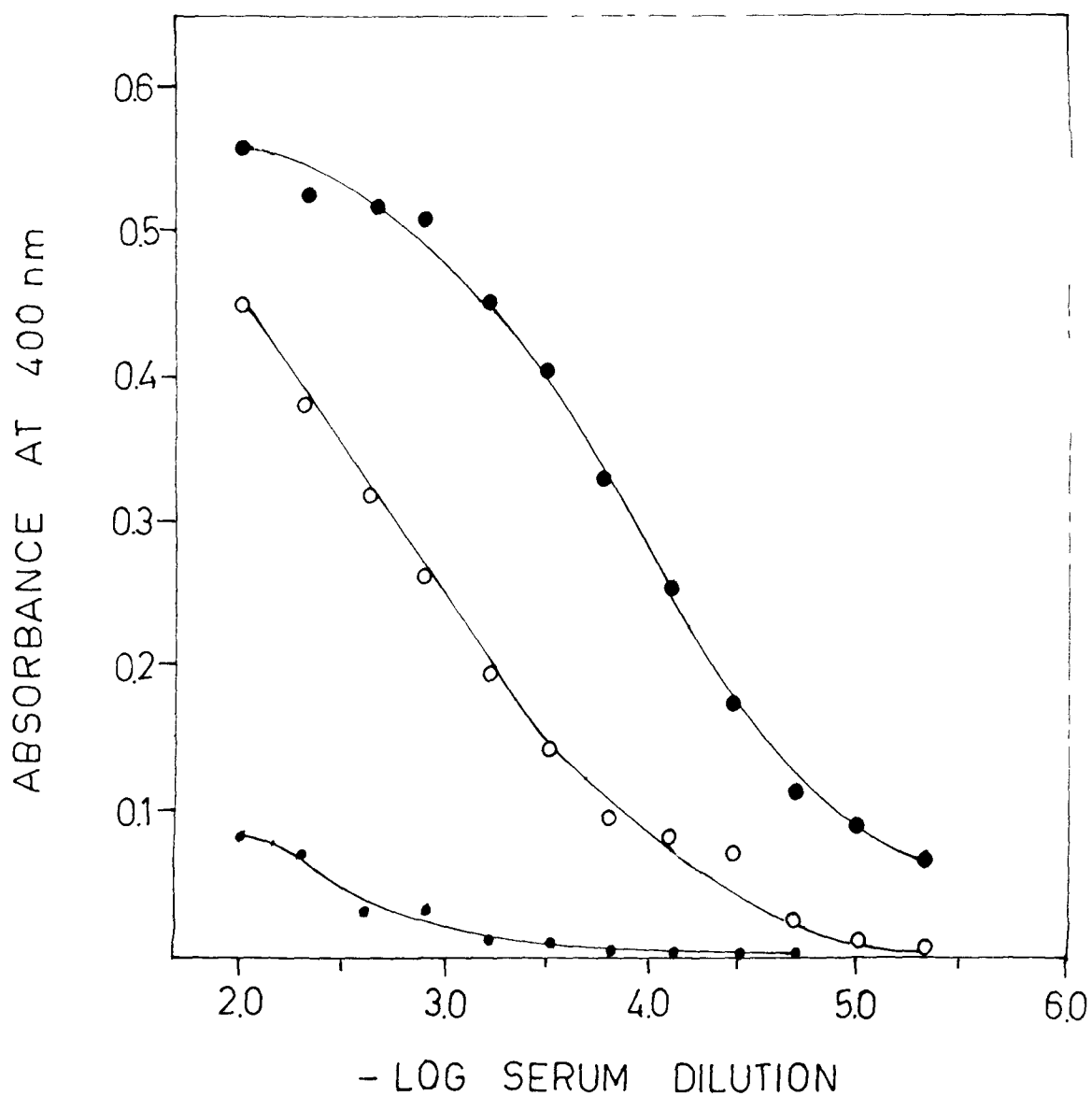


Fig.41. Binding of hCG by induced antibodies against alpha-psoralen complex and alpha subunit.

Preimmunized sera (—●—), alpha-subunit immunized sera (—●—) and alpha-psoralen complex immunized sera (—○—).

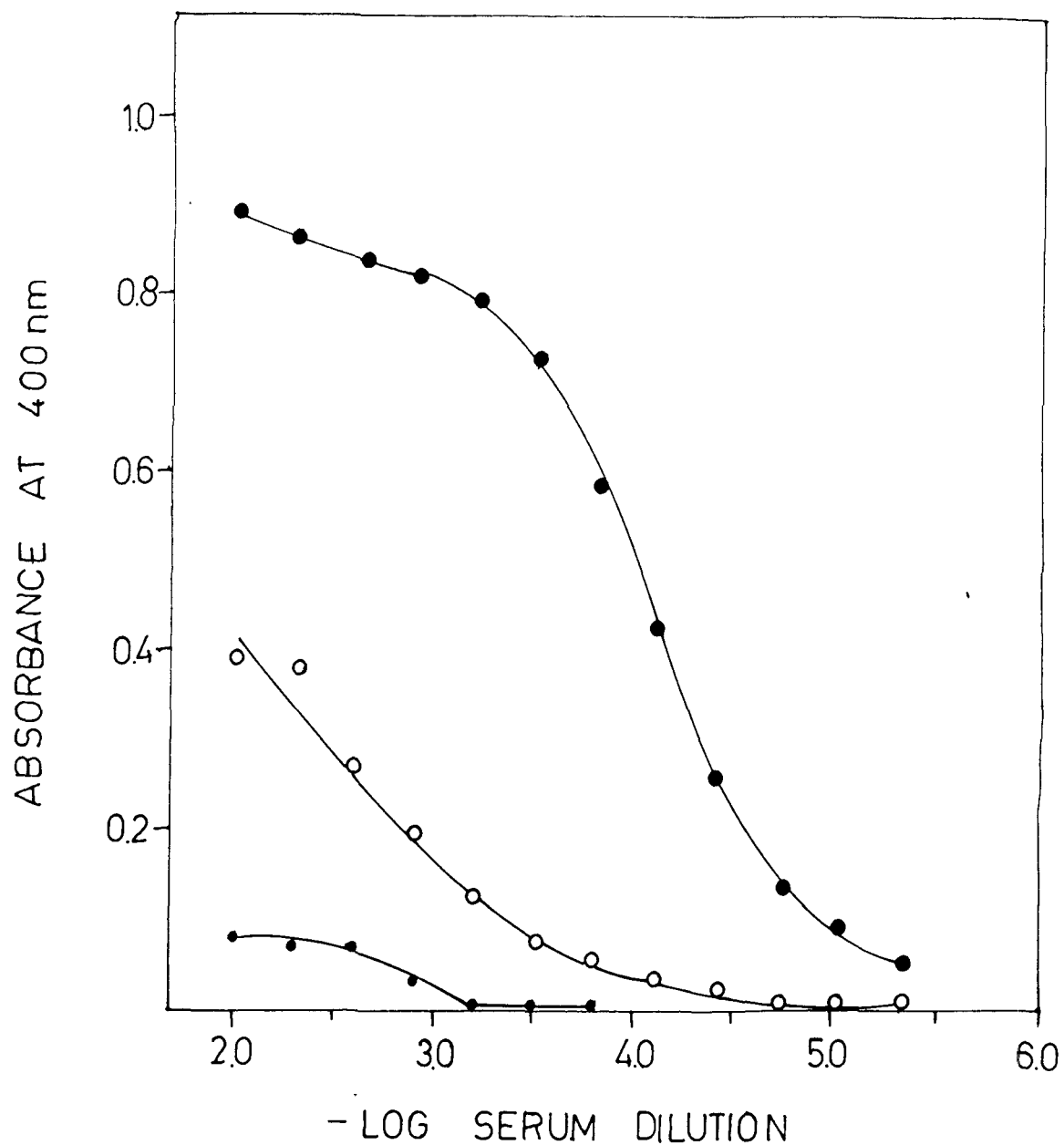


Fig.42. Binding of hCG by induced antibodies against beta-psoralen complex and beta subunit.

Preimmunized sera (—●—), beta-subunit immunized sera (—○—) and beta-psoralen complex immunized sera (—●—).

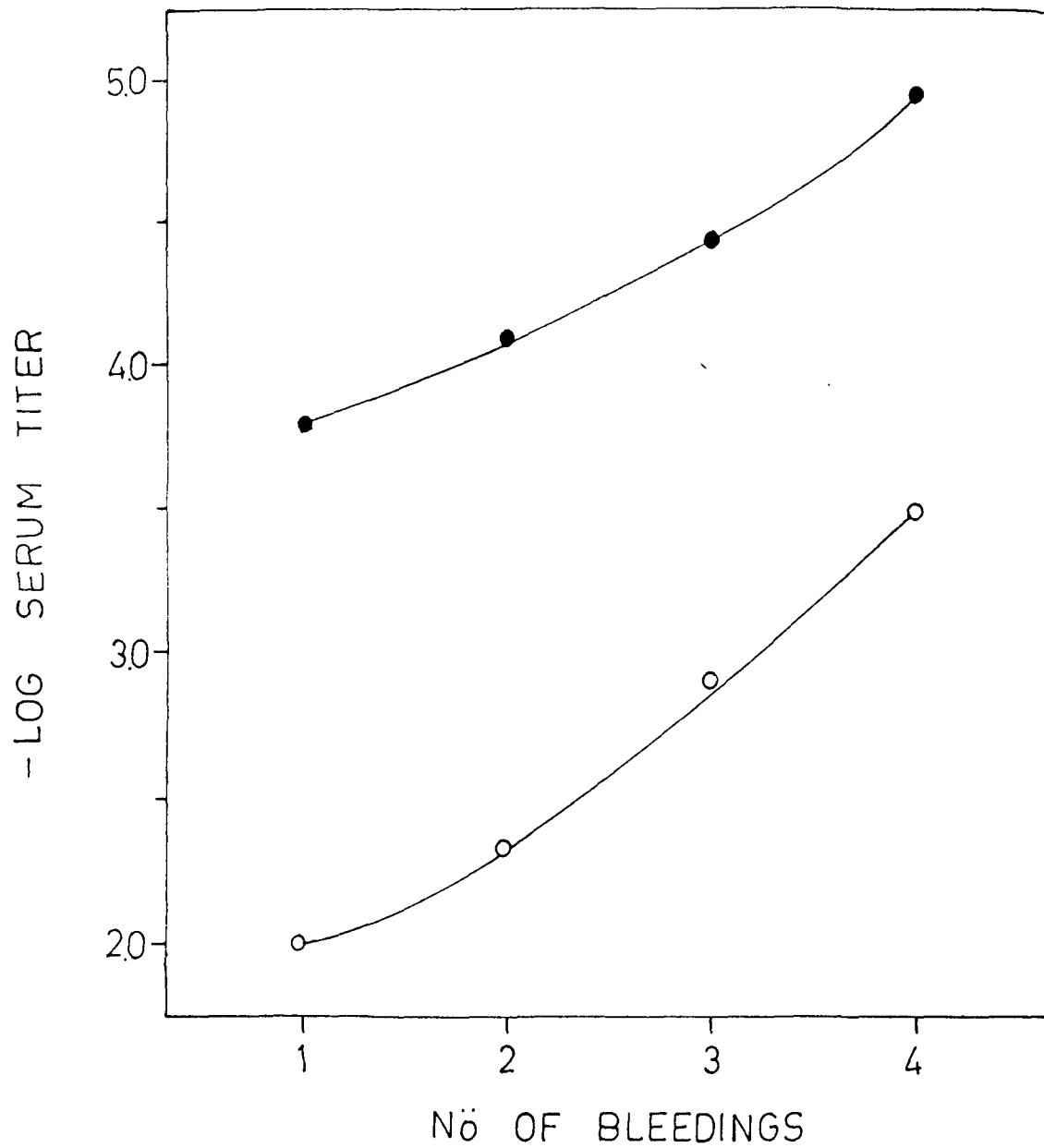


Fig.43. A comparative data of titers of induced antibodies against alpha-subunit and alpha-psoralen complex.

Alpha-subunit (—●—) and alpha-psoralen complex (—○—).

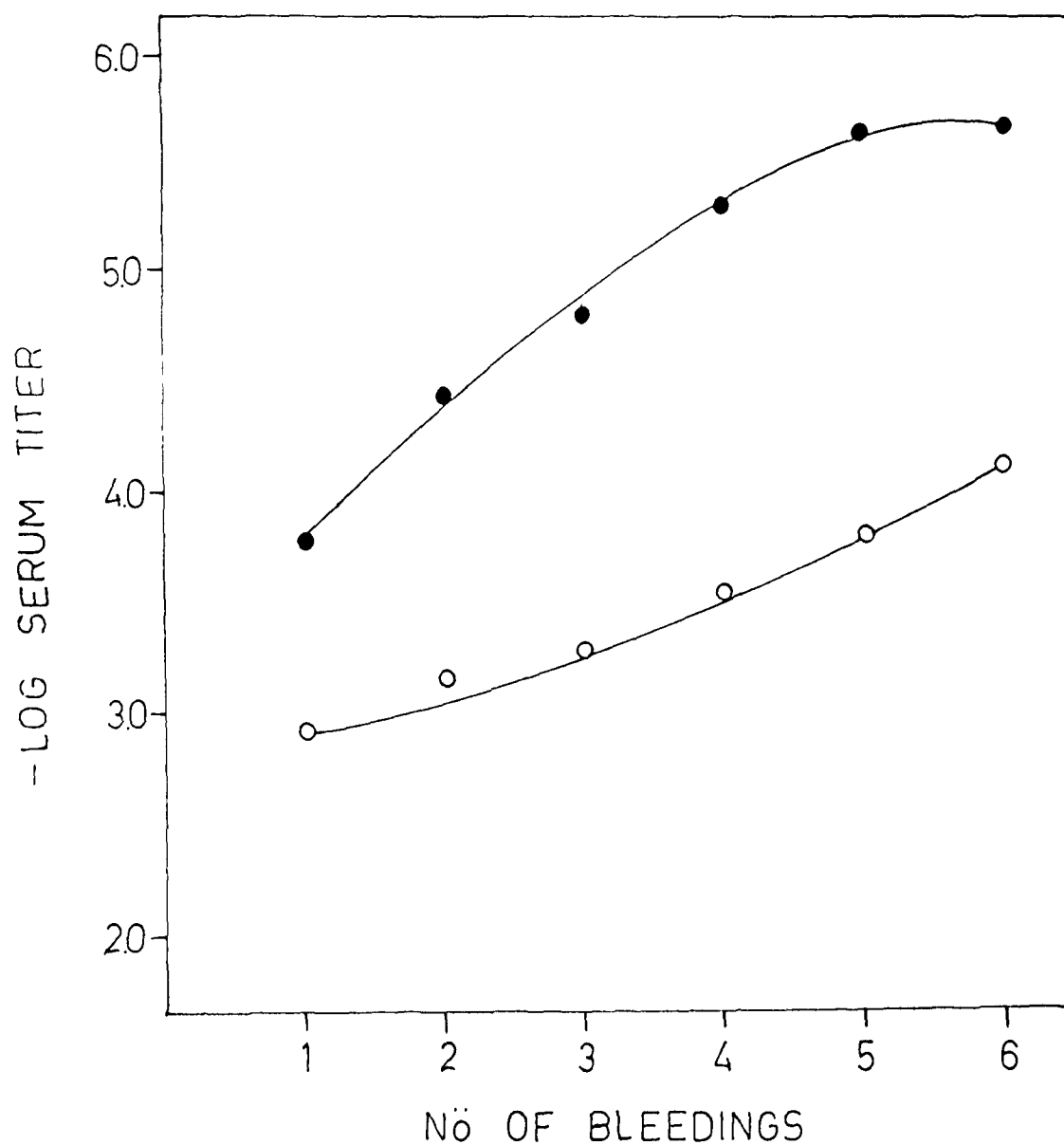


Fig.44. A comparative data of titers of induced antibodies against beta-subunit and beta-psoralen complex.

Beta-subunit (—●—) and beta-psoralen complex (—○—).

These peptides have been designated as PI, PII and PIII (Fig.45). The individual pooled fractions were concentrated and passed through a column of Sephadex G-50 (Fig.46). The peak fractions were pooled and concentrated and used in subsequent studies.

20. Binding of Anti-hCG Antibodies with Peptides

The binding of anti-hCG antibodies with peptides was studied by competition experiments (Fig.47). At a concentration of 100 μ g/ml of peptides, in hCG-anti-hCG-antibody activity, inhibition was observed to the extent of 66% for PI, 44%, for PII and no inhibition for PIII.

21. Binding of Anti-Beta-Subunit Antibodies with Peptides

The results of inhibition ELISA are shown in Fig.48. At a concentration of 100 μ g/ml, the inhibition was 52% for peptide I, 36% for peptide II and no inhibition for peptide III.

22. Affinity Isolation of Anti-Peptide IgG

Anti-PI antibodies were isolated from anti-hCG sera by affinity chromatography using a column of Sepharose 4B coupled with peptide I.

Affinity purified anti-PI antibodies were checked for reactivity with hCG, beta subunit and alpha subunit immobilized on polystyrene plates. The results showed maximum activity with

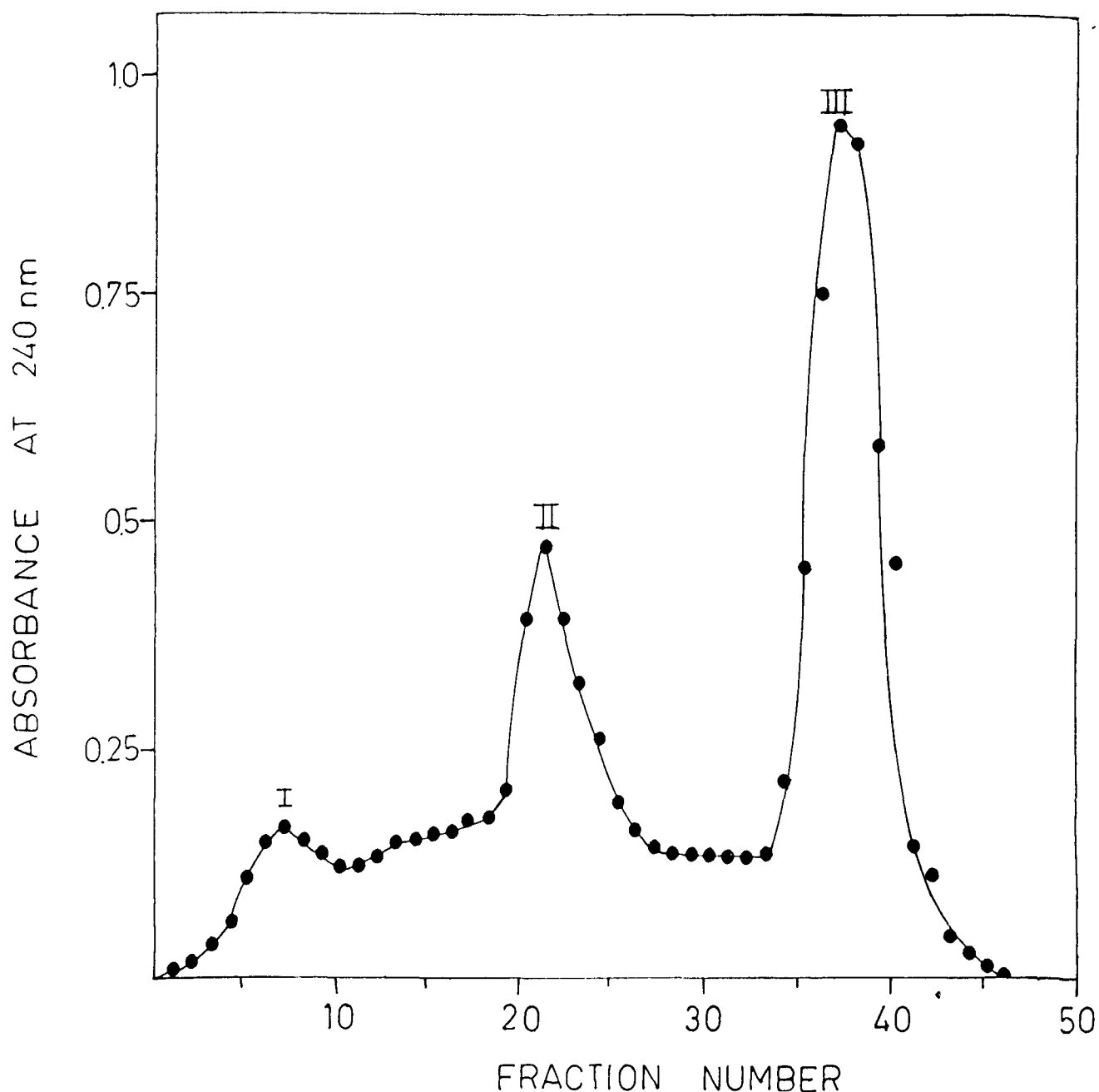


Fig.45. Gel filtration after hydrolysis of carboxymethylated beta-subunit by chymotrypsin on Sephadex G-25 column.

Column size: 1.5 x 60cm; eluent: 0.5M NaHCO_3 ;
 fraction size: 2.0 ml; flow rate: 15 ml/hr. Fraction under
 the peak I, II and III were pooled separately, concentrated
 and dialysed against the same eluent and further applied
 to a G-50 column.

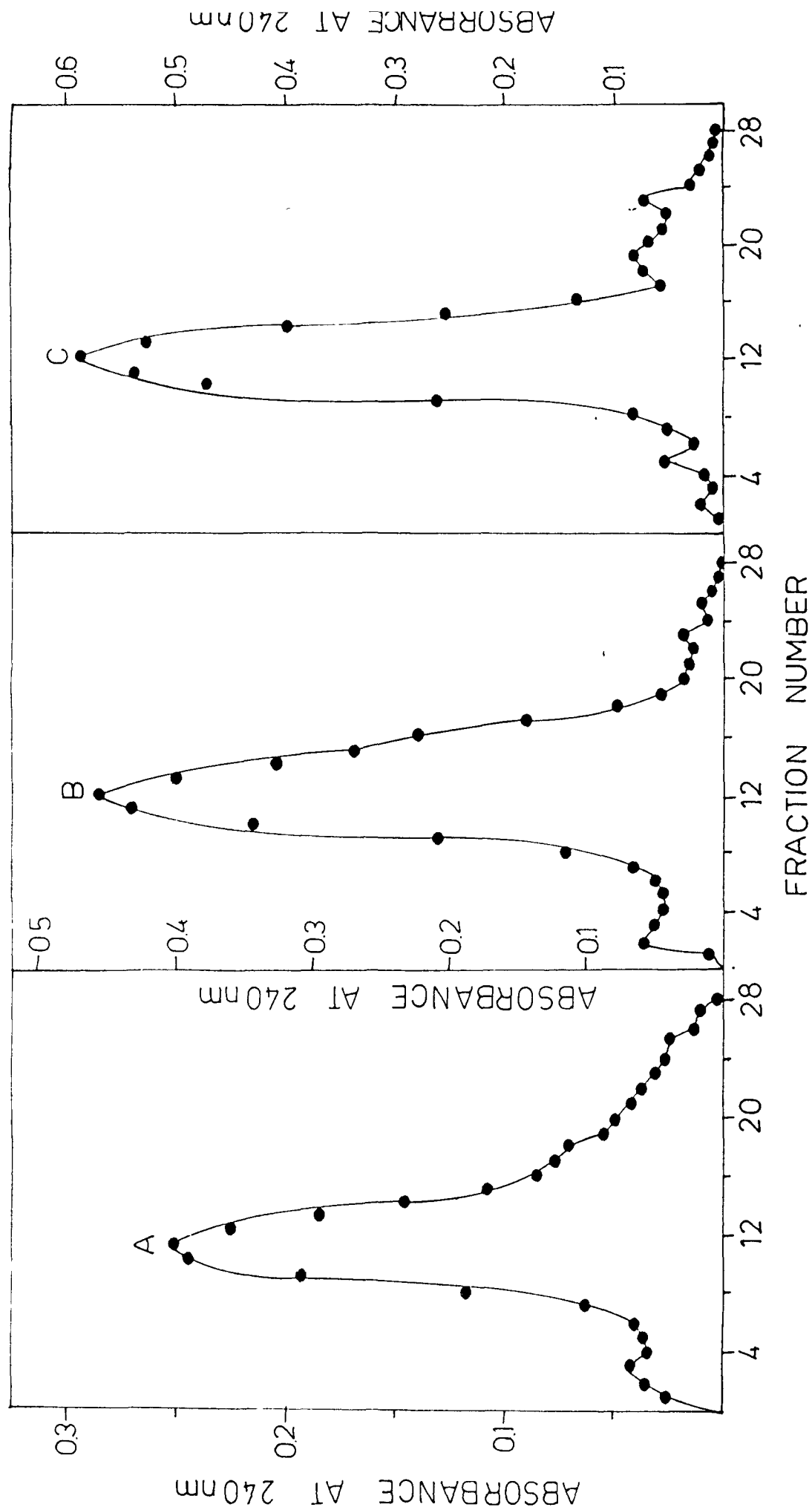


Fig.46. Purification of different peptides of beta-subunit on Sephadex G-50 column.

Column size: 1.8 x 55cm; eluent: PBS; flow rate: 20 ml/hr and fraction size:

2.5 ml. A = peptide I; B = peptide II, C = peptide III.

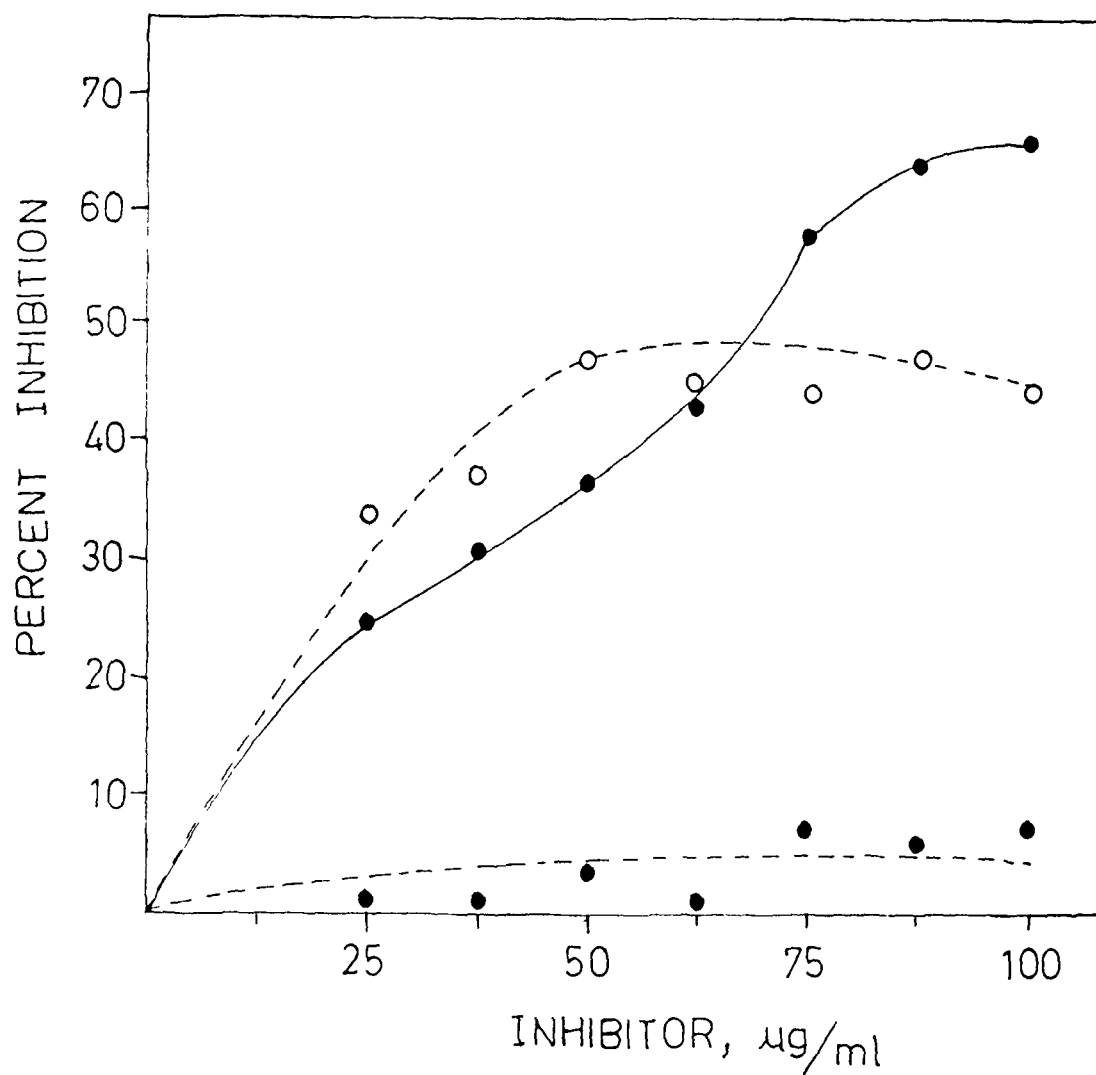


Fig.47. Inhibitory capacity of peptides of beta-subunit against anti-hCG antibodies.

Peptide I (—●—), peptide II (--○--)
and peptide III (---●---).

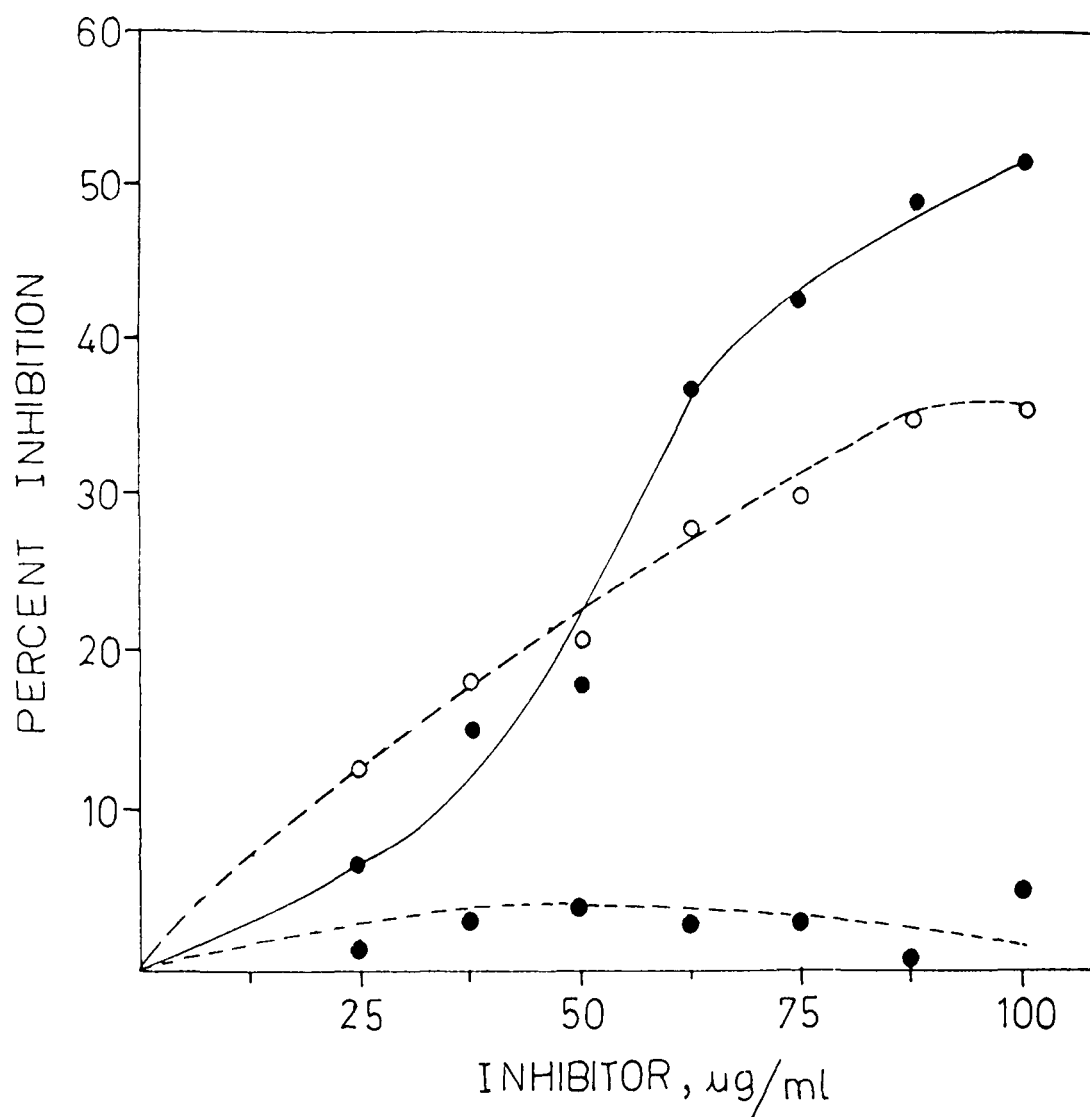


Fig.48. Inhibitory capacity of peptides of beta-subunit against anti-beta-hCG antibodies.

Peptide I (—●—), peptide II (---○---) and peptide III (---●---).

hCG followed by beta-subunit. With alpha subunit only a slight binding was observed (Fig.49).

When peptide I, peptide II and peptide III were coated on polystyrene plates, affinity purified IgG showed binding with peptide I, whereas peptide II and peptide III showed comparatively weak binding activity (Fig.50).

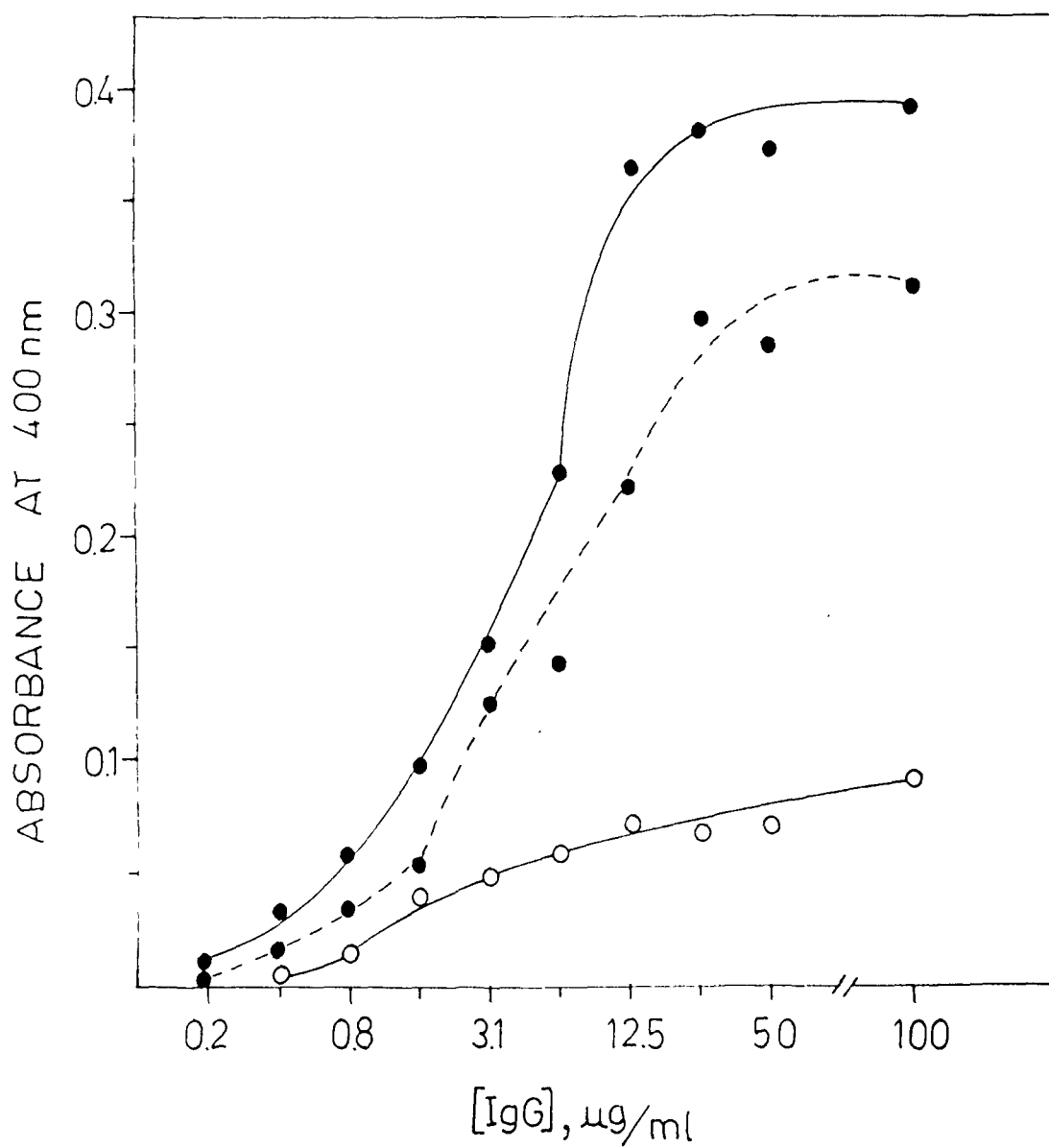


Fig.49. Reactivity of affinity purified anti-peptide I-IgG (anti-PI) with different antigens.

hCG (—●—), beta-subunit (---●---) and alpha-subunit (—○—).

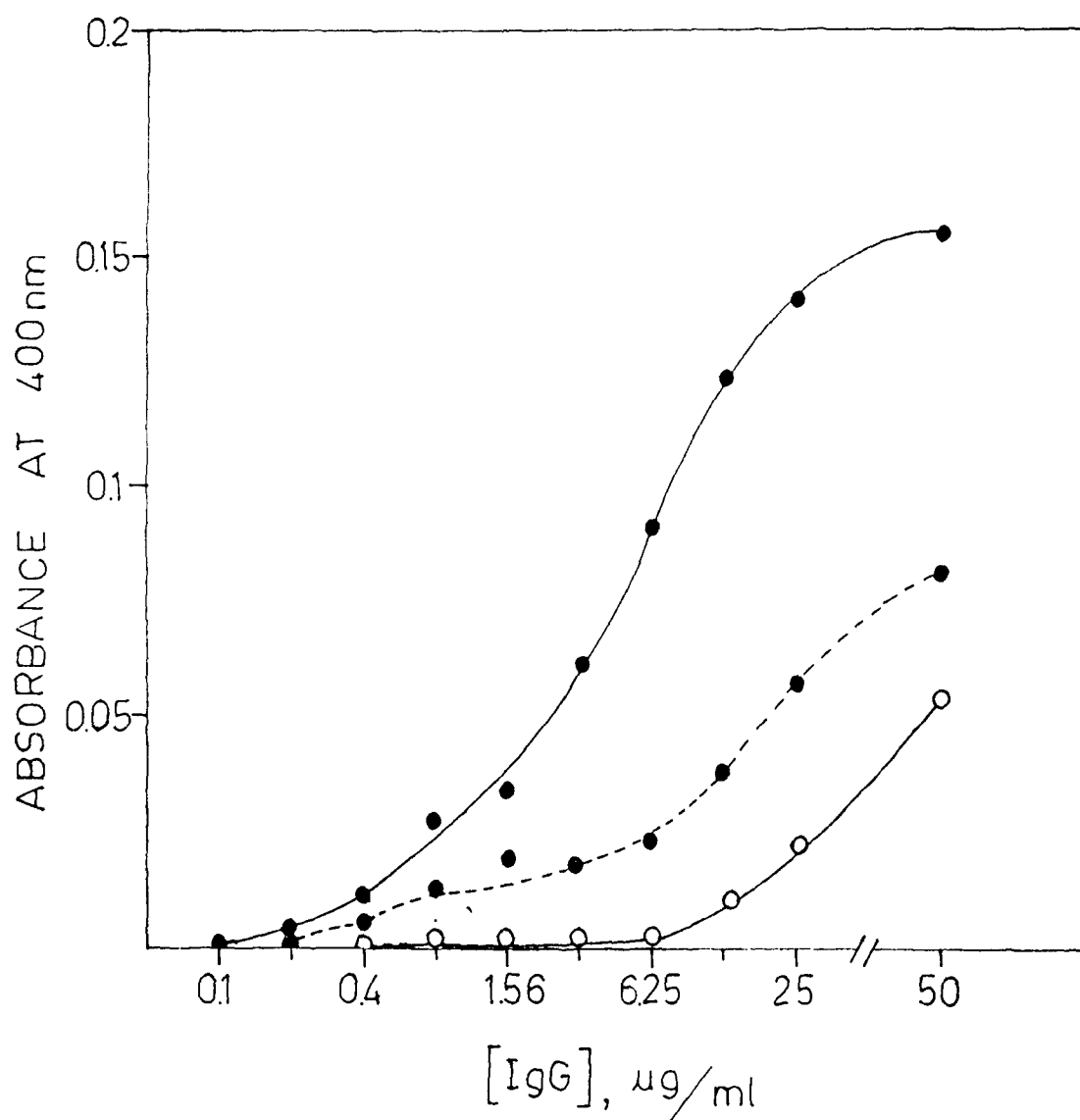


Fig.50. Reactivity of affinity purified anti-peptide I-IgG (anti-PI) with different peptide of beta-subunit.

Peptide I (—●—), peptide II (--●--)
and peptide III (—○—).

DISCUSSION

The concept of vaccine against pregnancy is an attractive approach to fertility control and the one which has received increasing attention in the last decade. The pituitary luteinizing hormone (LH) and chorionic gonadotropin (hCG) exercise a critical role in regulation of fertility of primates. In view of the high degree of homology between hCG and LH, it has been quite difficult to generate hCG specific antibodies that were generated against hCG. So the hCG and its subunits were modified in various ways (Stevens and Crystle, 1973; Pandian et al, 1980; Iwasa et al, 1981; Covey and Laurance, 1984; Caraux et al, 1985; Gupta et al, 1985) to minimize the cross reactivity against LH.

A number of physico-chemical studies have been performed in order to obtain information concerning the overall, conformation of hCG, but hitherto, the determination of the three dimensional structure of either the intact hormone or its free subunits has remained unsolved. Immunochemical methods have recently been utilized to define antigenic sites on the three dimensional surface of hCG and its subunits (Ehrlich et al, 1985; Schwarz et al, 1986). The major deterrent in the case of hCG has been the difficulty in its purification. Even highly purified preparations display microheterogeneity. However, the source of microheterogeneity has been recognized and is due to

the variation in their carbohydrate content and not in the polypeptide chain. The fluctuation in the carbohydrate units is probably due to their degradation or incomplete synthesis. It is consistent with the accepted view that the monosaccharides are attached stepwise to the completed polypeptide chain in the smooth endoplasmic reticulum by appropriate glycosyl transferase and sugar nucleosides (Spiro, 1970). It is therefore conceivable that the carbohydrate units would be at various stages of completion at the time of isolation of a glycoprotein.

The fluorescence intensity of proteins has been reported to be sensitive towards the structural perturbations induced in the molecule (Konev, 1967). Protein fluorescence is primarily due to the emission from tyrosine and tryptophan fluorophores. In spite of the presence of both the fluorophores, hCG exhibited a single emission peak of tyrosine at 350 nm and lacks the peak of tryptophan. The results are consistent with the observation that tryptophan residues are absent in hCG molecule (Morgan et al, 1975).

Absorbance at 275 nm of irradiated hCG decreased with increasing radiation dose. This may be ascribed either to the photodestruction of tryrosine residues or the exposure of these residues to hydrophillic environment upon unfolding of the hCG molecule. The decrease in absorbance of hCG is consistent with the destruction of these residues by quantitative analysis. Photodestruction of these residues resulted in the change in hormone conformation.

The changes in structure of the hormone induced by radiation have been detected by monitoring the susceptibility of structurally altered protein to proteolytic enzymes. In the present study, the resistance of native hCG towards tryptic attack is consistent with the fact that most of the protein in their native state remain unhydrolyzed by proteolytic enzyme due to non-availability of sites. However, after UV radiation, hCG became progressively susceptible to tryptic digestion. This marked difference in the behaviour of native and photoinactivated hCG indicates that these two forms of hormone have two different conformation. In the former case, the sites for tryptic attack are buried, while, in the later these were accessible to trypsin due to change in structure of the molecule. However, as the radiation dose increased, the susceptibility of hCG to trypsin decreased. This might be due to either the destruction of amino acids that are susceptible to digestion or there is a partial change in conformation of irradiated hCG molecule in such way that amino acids are no more accessible to trypsin digestion. The hormone contains 11 arginine and 7 lysine residues (Bahl, 1969) which contribute the possible sites for tryptic attack.

The influence of protein denaturant, urea, on the stability of hCG, showed that noncovalent bonds are barely sufficient to stabilize the native structure of hCG. The data on urea inactivation of native and irradiated hCG showed differences in their structural stability. Native hCG is more susceptible to

urea denaturation as compared to irradiated hCG as the photo-inactivated hCG needs high concentration of urea for complete unfolding. The difference might be due to the change in structure on account of irradiation.

The antibodies against irradiated hCG cross-react with hCG but appreciably less binding was noticed. During photo-inactivation, the native structure of hCG is probably disrupted, resulting in partial loss of immunogenicity. This was confirmed by immunohistological studies. Native hCG was biologically active and enhanced the process of spermatogenesis so as to the full maturation of sperms. However, photoinactivated hCG arrested spermatogenesis and the sperms did not reach maturation stage. These results confirm that immunogenicity and biological activity are associated with the native structure of hCG and UV irradiation has affected the native structure of the molecule with decreased biological activity.

The evaluation of the percentage of psoralen bound in vitro to hCG was evaluated by operating the experiment under physiological conditions with regard to pH and ionic strength (Veronese et al, 1978). A direct evaluation of the binding of psoralen to hCG by usual methods, such as ultrafiltration by Amincon centriflow membrane or under pressure by Diaflo membrane were not feasible because 8-MOP has been reported to bind strongly to these membrane filters (Veronese et al, 1978).

Reliable data were obtained by equilibrium dialysis and fluorescence quenching techniques. The study of quenching of extrinsic as well as intrinsic bound fluorophores has proved to have great significance in studying the ligand-protein interaction (Eftink and Ghiron, 1981). This analysis is shown in terms of Scatchard plot based on the fact that intrinsic fluorescence of psoralen was quenched significantly after interaction with hCG.

A slight curvature in the binding data indicated two possible modes of binding (a) increase in psoralen concentration restricts its further interaction with hCG (b) low affinity sites are also involved in the binding reaction.

The data obtained by fluorescence quenching indicated four binding sites for psoralen on hCG with $K_a = 0.795 \times 10^5 \text{ M}^{-1}$. Preliminary data of equilibrium dialysis yielded a value of $K_a = 0.33 \times 10^5 \text{ M}^{-1}$ showing a definite correlation between these two altogether different techniques. Effective competition was observed between anti-hCG antibodies and psoralen for the binding sites on hCG molecule. The results clearly indicate that there was inhibition in the immunoreactivity of hCG-anti-hCG system by the psoralen. This revealed that the psoralen binding sites and epitope might be identical or recognizing the same amino acid(s) on the hCG molecule.

Immunological studies on hCG-psoralen complex has suggested that binding of psoralen to hCG results in enhanced immunogenicity.

Anti-hCG-psoralen antibodies bind hCG with high affinity as compared to anti-hCG antibodies. Therefore, the neutralizing capacity of anti-hCG-psoralen antisera would be greater as compared to unbound hCG. The reaction was found to be specific as is shown by inhibition experiments.

The immunological studies on the subunit and their complexes with psoralen showed that subunits after binding with psoralen became less immunogenic and induced decreased amount of antibodies as compared to anti-subunit antibodies. The decreased antigenicity might be due to either nonavailability of epitopes due to psoralen binding in the near vicinity of the antigenic sites or the binding of psoralen to a key amino acid of antigenic site rendering it less immunogenic.

Amino acid sequences of the β -subunits of hCG and LH had marked difference between them. The β -subunit of hCG contains not only the unique carboxy-terminal 30 residue peptide but also some other sequences in the rest of the molecule. The immunologic properties of the synthetic carboxy-end of β -hCG but smaller than the natural fragment have been reported. These sequences have prepared both by chemical synthesis (Schneider et al, 1975; Matsuura et al, 1978; Caraux et al, 1985) and by enzymatic cleavage (Louvét et al, 1974). In the present study, we have used the chymotryptic digested three peptides of β -subunit. The antibodies against peptide I (PI) from anti-hCG serum were isolated on the column of Sepharose-4B covalently linked to PI.

The isolated antibodies against PI showed binding with hCG as well as with β hCG but exhibited low level of reactivity with α hCG. It appears that the antigenic determinants of PI are a part of hCG and β hCG but not of α hCG. Moreover, the antibodies did not show binding towards polypeptides PII and PIII of lower molecular weight. The antibodies are thus specific for PI.

The recognition of antibodies induced against hCG and β hCG with a series of enzymatically digested peptide clearly indicates that the antibody recognition sites of hCG/ β hCG reside within PI and PII. The results show among these two peptides the major reactivity is present in peptide PI with low reactivity in PII. While the antigenic determinants on the surface of the hCG molecule have not been thoroughly defined in structural terms, it is clear that despite the great similarity of amino acid sequences of β -subunit, there is at least one conformationally determined epitope that is relatively specific for hCG (Vaitukaitis et al, 1972; Birken et al, 1980; Pandian et al, 1980).

In conclusion, this study clearly indicates that photo-inactivation caused altered native structure of hCG molecule which results in decreased immunological and biological properties. Furthermore the binding of psoralen to hCG and its subunits was confirmed and evaluated by fluorescence quenching experiments. Although, it is difficult to locate the exact binding position of psoralen of hCG but epitope and binding site appears to be similar. The studies on peptides of β -subunit showed that major reactivity of hCG and β -hCG was present in PI with somewhat low reactivity in PII.

R E F E R E N C E S

- Amr, S., Wehmann, R.E., Birken, S., Canfield, R.E. and Nisula, B.C.
(1983) J. Clin. Invest. 71, 329.
- Arnon, R. (1973) In: 'The Antigens' (ed., M. Sela) Vol.I, pp. 87,
Academic Press, New York.
- Artuc, M., Stuetzgen, G., Schalla, W., Schalfer, M. and Gazith, J.
(1979) Br. J. Dermatol. 101, 669.
- Ashitaka, Y. and Koide, S.S. (1974) Fertil. Steril. 25, 177.
- Asif, M., Ali, R. and Husain, I. (1969) Indian J. Biochem. 6, 99.
- Atassi, M.Z. (1975) Immunochemistry 12, 423.
- Atassi, M.Z. (ed.) (1977-1979) " Immunochemistry of Proteins"
Vol.1-3, Plenum Press, New York.
- Ayala, A.R., Saad, A., Vazquez, X., Ramirez-Wiella, G. and
Perches, R.D. (1983) Am. J. Reprod. Immunol. 3, 149.
- Bagshawe, K.D., Orr, A.H. and Rushworth, A.G.J. (1968) Nature
217, 950.
- Bagshawe, K.D. (1969). " Choriocarcinoma: The clinical Biology
of the Trophoblast and its Tumours" . Edward Arnold,
London.
- Bahl, O.P. (1969a) J. Biol. Chem. 244, 575.

- Bahl, O.P. (1969b) J. Biol. Chem. 244, 567.
- Bahl, O.P. (1971) In: "Structure-Activity Relationships of Proteins and Polypeptide Hormones" (eds., M. Margoulies and F.C. Greenwood) Part I, pp. 99, Excerpta Medica, Amsterdam.
- Bellisario, R., Carlsen, R.B. and Bahl, O.P. (1973) J. Biol. Chem. 248, 6796.
- Begent, R.H., Searle, F., Stanway, G., Jewkes, R.F., Jones, B.E., Vernon, P. and Bagshawe, K.D. (1980) J. Roy. Soc. Med. 73, 624.
- Bellet, D.H., Bidart, J.M., Ozturk, M., Troalen, F., Ghillant, P., Pernas, P., Wands, J.R. and Bohuon, C.J. (1987) Nucl. Med. Biol. 14, 295.
- Berger, P., Kofler, R. and Wick, G. (1984). Am. J. Reprod. Immunol. 5, 157.
- Bhalla, V.K., Haskell, J., Grier, H. and Mahesh, V.B. (1976) J. Biol. Chem. 251, 4947.
- Birken, S., Canfield, R.E., Lauer, R., Agosto, G.M. and Gabel, M. (1980) Endocrinology 106, 1659.
- Birken, S., Canfield, R., Agosto, G. and Lewis, J. (1982) Endocrinology 110, 1555.
- Braustein, G.D., Vaitukaitis, J.L. and Ross, G.T. (1972) Endocrinology 91, 1030.

- Braustein, G.D., Vogel, C.L., Vaitukaitis, J.L. and Ross, G.T.
(1973) *Cancer* 32, 223.
- Bychkov, V., Rothman, M. and Bardawil, W.A. (1983) *Am. J. Clin. Pathol.* 79, 414.
- Canfield, R.E., Bell, J.J. and Agosto, G.M. (1970) In:
"Gonadotropins and Ovarian Development" (eds., W.R. Butt,
A.C. Crooke and M. Ryle) pp. 161, Livingstone, Edinburgh.
- Canfield, R.E., Morgan, F.J., Kammerman, S., Bell, J.J. and
Agosto, G.M. (1971) *Rec. Prog. Horm. Res.* 27, 121.
- Canfield, R.E., Birken, S., Morse, J.H. and Morgan, F.J. (1976)
In: "Peptide Hormones" (ed., J.A. Parsons) pp. 299,
MacMillan, London.
- Caraux, J., Chichehian, B., Gestin, C., Longhi, B., Lee, A.C.,
Powell, J.E., Stevens, V.C. and Pourquier, A. (1985)
J. Immunol. 134, 835.
- Carlsen, R.B., Bahl, O.P. and Swaminathan, N. (1973) *J. Biol. Chem.* 248, 6810.
- Catt, K.J. and Dufau, M.L. (1973) In: "Receptors for
Reproductive Hormones" (eds., B.W. O'Malley and A.R. Means)
pp. 379, Plenum Press, New York.
- Catt, K.J., Tsuruhara, T., Mendelson, C., Ketelegers, J.M. and
Dufau, M.L. (1974) In: "Hormone Bindings and Target Cell
Activation in the Testis" (eds., M.L. Dufau and A.R. Means)
pp. 1, Plenum Press, New York.

- Campbell, D.G., Brown, J.B., Fortune, D.W., Pepperell, R. and Beischer, N.A. (1970) J. Obstet, Gynecol. Br. Cwlth. 77, 410.
- Carlsen, R.B., Bahl, O.P. and Swaminathan, N. (1973) J. Biol. Chem. 248, 6810.
- Channing, C.P. and Kammerman, S. (1974) Biol. Reprod. 10, 179.
- Chang, C.C., Tsong, Y.Y., Rone J.D., Segal, S.J., Chang, D., Leban, J. and Folkers, K. (1981) Fertil. Steril, 36, 659.
- Chang, C.C., Nash, H.A. and Tsong, Y.Y. (1986) J. Reprod. Immunol. 9, 327.
- Chen, H.C., Hodgen, G.D., Matsura, S., Lee, L.J., Gross, E., Reichert, L.E., Birken, S., Canfield, R.E. and Ross, G.T. (1976) Proc. Natl. Acad. Sci. USA 73, 2885.
- Chow, S.N., Hsich, C.Y., Ouyang, P.C., Leung, W.Y., Yuen, B.H. and Lee, C.T.G. (1986) Diagn. Immunol. 4, 194.
- Cinader, B. (ed.) (1967) "Antibodies to Biologically Active Molecules" . Permagon, Oxford.
- Covey, D.C. and Laurence, K.A. (1984) Am. J. Reprod. Immunol. 5, 114.
- Cotton, R.G.H. (1985) Med. Res. Rev. 5, 97.
- Crosignani, P.G., Brambati, B. and Nencioni, T. (1971) Am. J. Obstet. Gynecol. 109, 985.

- Crosignani, P.G., Nencioni, T. and Brambati, B. (1972) J. Obstet. Gynecol. Br. Cwlth. 79, 122.
- Delfs, E. (1959) Ann. N.Y. Acad. Sci. 80, 125.
- Diczfalusy, E. (1953) Acta. Endocr. Copenh. 12, 7.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Robers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350.
- Eftink, M.R. and Ghiron, C.A. (1981) Anal. Biochem. 114, 199.
- Ehrlich, P.H. and Canfield, R.E. (1982) J. Immunol. 128, 2709.
- Ehrlich, P.H., Moyle, W.R. and Canfield, R.E. (1985) In: "Methods in Enzymology", (eds., L. Birnbaumer and B.W. O'Malley) pp. 638, Academic Press, New York.
- Ernest, M.J. and Kim, K.H. (1974) J. Biol. Chem. 249, 6770.
- Falman, C., Ryan, R.J., Zwirek, S.J. and Rubin, M.E. (1968) J. Clin. Endocr. Metab. 28, 1323.
- Folin, O. and Ciocalteu, V. (1927) J. Biol. Chem. 73, 627.
- Fowlks, W.L. (1959) J. Invest. Dermatol. 32, 233.
- Franchimont, P. and Reuter, A. (1972) In: "Structure Activity Relationships of Protein and Polypeptide Hormones" Part II, pp. 381, Excerpta Medica, Amsterdam.
- Friedman, M.H. and Weinstein, G.L. (1937) Endocrinology 21, 489.

- Fusco, F.D. and Rosen, S.W. (1965) *New Engl. J. Med.* 275, 507.
- Furuhashi, Y., Mano, H., Hattori, S., Goto, S. and Tomada, Y.
(1983) *Nippon Sanka Fujinka Gakkai Zasshi* 35, 2415.
- Gastineau, C.F., Albert, A. and Randall, L.M. (1949) *J. Clin. Endocr. Metab.* 9, 615.
- Geiger, W., Kaiser, R. and Franchimont, P. (1971) *Acta Endocr. Copenh.* 68, 169.
- Goldstein, D.P., Kosasa, T.S. and Skarim, A.T. (1974) *Surg. Gynecol. Obstet.* 138, 747.
- Goldenberg, D.M., Kim, E.E. and Deland, F.H. (1981) *Proc. Natl. Acad. Sci. USA*, 78, 7754.
- Got, R., Bourrillon, R. and Michon, J. (1960) *Bull. Soc. Chim. Biol.* 42, 41.
- Goverde, B.C., Veenkamp, F.J.N. and Homan, J.D.H. (1968) *Acta Endocr. Copenh.* 59, 105.
- Gros, C. and Labouesse, B. (1969) *Eur. J. Biochem.* 7, 463.
- Gupta, S.K. and Talwar, G.P. (1980) *Indian J. Exp. Biol.* 18, 1361.
- Gupta, S.K., Singh, O.M. and Talwar, G.P. (1985) *Am. J. Reprod. Immunol.* 7, 104.
- Hallick, L.M., Yokota, H.A., Bartholomew, J.C. and Hearst, J.E.
(1978) *J. Virol.* 27, 127.

- Hamberger, C. (1944) Acta Obstet. Gynecol. Scand. 24, 45.
- Han, S.S., Rajaniemi, H.J., Cho, M.I., Hirshfield, A.M. and Midgley, A.R.Jr. (1974) Endocrinology, 95, 589.
- Haour, F. and Saxena, B.B. (1974) J. Biol. Chem. 249, 2195.
- Hearn, J.P., Short, R.V. and Lunn, S.F. (1975) In: "Physiological Effects of Immunity against Reproductive Hormones" (eds., R.G. Edwards and M.H. Johnson) pp. 229, Cambridge University Press, London.
- Hirs, C.H.W. (ed.) (1967) "Methods in Enzymology" . Academic Press, New York.
- Hisley, J.C. and Fesche, H. (1966) Proc. Soc. Exp. Biol. Med. 121, 235.
- Hulme, M.J., Fehilly, C.G. and Hearn, J.P. (1980) J. Reprod. Immunol. 1, 329.
- Hum, V.G., Mnipfel, J.W. and Mori, K.F. (1974) Biochemistry 13, 2359.
- Hum, V.G., Botting, H. and Mori, K.F. (1976) Endocr. Res. Commun. 3, 145.
- Ito, H. and Tahara, E. (1983) Acta Pathol. Jpn. 33, 287.
- Iwasa, S., Kitada, C., Yoshida, I., Kondo, K., Horí, M. and Fujino, M. (1981) J. Biochem. 89, 1091.

- Jacobson, G.K. and Jacobson, M. (1983) *Acta Pathol. Microbiol. Immunol. Scand.* 91, 165.
- Javadpour, N., Utz, M. and Soares, T. (1980) *J. Urol.* 124, 615.
- Khan, C.R., Rosen, S.W., Weintraub, B.D., Fajans, S.A. and Gorden, P. (1977) *New Engl. J. Med.* 297, 565.
- Kaufman, K.D. (1960) *J. Org. Chem.* 26, 117.
- Kennedy, J.F. and Chaplin, M.F. (1976) *Biochem. J.* 155, 303.
- Khazaeli, M.B., England, B.G., Dieterle, R.C., Nordblom, G.D., Kabza, G.A. and Beierwaltes, W.H. (1981) *Endocrinology* 109, 1290.
- Kido, I. (1937) *Zentralbl. Gynak.* 61, 1551.
- Kofler, R., Berger, P. and Wick, G. (1982) *Am. J. Reprod. Immunol.* 2, 212.
- Konev, S.V. (1967) In: "Fluorescence and Phosphorescence of Proteins and Nucleic acids" . (ed., S. Udenfriend) pp.9, Plenum Press, New York.
- Kurata, N. and Tan, E.M. (1976) *Arth. Rheum.* 19, 574.
- Laemmli, U.K. (1970) *Nature* 227, 680.
- Landefeld, T.D., McWilliam, D.R. and Boime, I. (1976) *Biochem. Biophys. Res. Commun.* 72, 381.
- Lee, C.Y. and Ryan, R.J. (1971) *Endocrinology* 89, 1515.

- Lee, C.Y. and Ryan, R.J. (1972) Proc. Natl. Acad. Sci. USA, 69, 3520.
- Lerman, S. and Brokman, R.F. (1978) Interdiscip. Top. Gerontol. 13, 154.
- Lerman, S., Megaw, J. and Willis, I. (1980) Photochem. Photobiol. 31, 235.
- Longeon, A., Henry, J.P. and Henry, R. (1986) J. Immunol. Methods, 89, 103.
- Louvet, J.P., Ross, G.T., Birken, S. and Canfield, R.E. (1974) J. Clin. Endocrinol Metab. 39, 1155.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.
- Mason, T.E., Phifer, R.F., Spicer, S.S., Swallow, R.A. and Dreskin, R.B. (1969) J. Histochem. Cytochem. 17, 563.
- Masure, H.R., Jaffe, W.L., Sickel, M.A., Birken, S., Canfield, R.E. and Vaitukaitis, J.L. (1981) J. Clin. Endocrinol. Metab. 53, 1014.
- Matsuura, S., Chen, H.C. and Hodgen, G.D. (1978) Biochemistry, 17, 575.
- Megaw, J. and Lerman, S. (1980) Am. Soc. Photobiol. Meeting. Feb., 118
- Midgley, A.R. and Pierce Jr., G.B. (1962) J. Exp. Med. 115, 289.

- Midgley, A.R., Fong, I.F. and Jaffe, R.B. (1967) *Nature* 213, 733.
- Mizuchi, A., Kitagawa, N., Miyachi, Y., Lio, M. (1984) *J. Immunol. Methods*, 74, 369.
- Moore, S. and Stein, W.H. (1948) *J. Biol. Chem.* 176, 367.
- Morinaga, S., Ojima, M. and Sasano, N. (1983) *Cancer* 52, 1281.
- Mori, K.F. (1970) *Endocrinology* 86, 97.
- Morgan, F.J., Birken, S. and Canfield, R.E. (1974) In: "Gonodotropins and Gonadal Functions" (ed., N.R. Moudgal) pp. 79, Academic Press, London.
- Morgan, F.J., Birken, S. and Canfield, R.E. (1975) *J. Biol. Chem.* 250, 5247.
- Morrison, R.T., Lyster, D.M., Alcorn, L.N., Rhodes, B.A.
Breslow, K. and Burchiel, S.W. (1984) *Clin. Nucl. Med.* 9, 20.
- Moudgal, R.N., Mukku, V.R., Prahalada, S., Murty, G.S.R.C. and Li, C.H. (1978) *Fertil. Steril*, 30, 223.
- Moyle, W.R., Ehrlich, P.H. and Canfield, R.E. (1982) *Proc. Natl. Acad. Sci. USA*, 79, 2245.
- Moyle, W.R., Anderson, D.M. and Ehrlich, P.H. (1983) *J. Immunol.* 131, 1900.

- Musch, K., Wolf, A.S. and Lauritzen, C. (1981) Clin. Chim. Acta 113, 95.
- Nagainis, P.A., Nakagawa, C.H., Baron, S.L., Fuller, S.A., Chandler, H.M. and Hurrell, G.R. (1986) Clin. Chim. Acta 160, 273.
- Nass, M.M.K. (1979) J. Cell Biol. 83, 358.
- Nash, H., Talwar, G.P. Segal, S., Luukkainen, T., Johansson, E.D.B., Vasquez, J., Coutinho, E. and Sundaram, K. (1980) Fertil. Steril. 34, 328.
- Nash, H.A., Chang, C.C. and Tsong, Y.Y. (1985) J. Reprod. Immunol. 7, 151.
- Nishimura, R., Endo, Y., Tanabe, K., Ashitaka, Y. and Tojo, S. (1981) J. Endocrinol. Invest. 4, 349.
- Nieschlog, E., Bernitz, S. and Tropert, M. (1982) Clin. Endocrinol. 16, 483.
- Ogiso, T., Tamura, S., Kato, Y. and Sugiura, M. (1974) J. Biochem. 76, 147.
- Okudaira, Y., Hashimoto, T., Hamanaka, N. and Yoshinara, S. (1971) J. Electron Microsc. 20, 93.
- Paik, W.K. and Kim, S. (1972) Biochemistry 11, 2589.
- Pandian, M.R., Bahl, O.P. and Segal, S.J. (1975) Biochem. Biophys. Res. Commun. 64, 1199.

- Pandian, M.R., Mitra, R. and Bahl, O.P. (1980) *Endocrinology* 107, 1564.
- Parham, P., Androlewicz, M.J., Brodsky, F.M., Holmes, N.J. and Ways, J.P. (1982) *J. Immunol. Methods* 53, 133.
- Pierce, J.G., Bahl, O.P., Cornell, J.S. and Swaminathan, N. (1971a) *J. Biol. Chem.* 246, 2322.
- Pierce, J.G., Liao, T.H., Howard, S.M., Shome, B. and Cornell, J.S. (1971b) *Rec. Prog. Horm. Res.* 27, 165.
- Plapp, B.V., Moore, S. and Stein, W.H. (1971) *J. Biol. Chem.* 246, 939.
- Poppe, W. and Grossweiner, L.I. (1975) *Photochem. Photobiol.* 22, 217.
- Rajaniemi, H.J., Hirshfield, A.N. and Midgley, A.R. (1974) *Endocrinology* 95, 557.
- Rao, C.V. (1973) In: "Symposium on Advances in Chemistry, Biology and Immunology of Gonadotropins. (Bangalore)
- Rao, C.V. and Saxena, B.B. (1973) *Biochim. Biophys. Acta* 313, 372.
- Rizkallah, T., Gurside, E. and Van de wiek, R.L. (1969) *J. Clin. Endocr. Metab.* 29, 92.
- Romeu, A.M., Martino, E.E. and Stoppani, O.M. (1976) *Biochim. Biophys. Acta* 439, 175.

- Ross, G.T. (1977) Am. J. Obstet. Gynecol. 129, 795.
- Scheider, C.H., Blaser, K., Pheuti, C. and Gurden, E. (1975)
FEBS Lett. 5, 272.
- Schwarz, S., Berger, P. and Wick, G. (1986) Endocrinology 118,
189.
- Scott, B.R., Pathak, M.A. and Mohn, G.R. (1976) Mutat. Res. 39,
29.
- Searle, F., Partridge, C.S., Kardana, A., Green, A.J., Buckley,
R.G., Bengent, R.H. and Rawlins, G.A. (1984) Int. J.
Cancer 33, 429.
- Sharpe, R.M. (1976) Nature 264, 644.
- Shahni, S.M., Kulkarni, P.P., Patel, K.L., Salahuddin, M. Das, C.
and Talwar, G.P. (1982) Contraception 25, 421.
- Shen, C.K.J. and Hearst, J.E. (1979) Anal. Biochem. 95, 108.
- Smith, E.B., Szulman, A.E., Hinshaw, W., Tyrey, L., Surti, V.
and Hammond, C.B. (1984) Am. J. Obstet. Gynecol. 149, 129.
- Song, P.S. and Ou, C.N. (1980) Ann. N.Y. Acad. Sci. 346, 355.
- Spiro, R.G. (1970) Ann. Rev. Biochem. 39, 599.
- Stahli, C., Stachelin, T., Miggiano, V., Schmidt, J. and
Haring, P. (1980) J. Immunol. Methods 32, 297.
- Stanworth, D.R. (1960) Nature 188, 156.

- Stevens, V.C. (1973) *Obstet. Gynecol.* 42, 496.
- Stevens, V.C. and Crystle, C.D. (1973) *Obstet. Gynecol.* 42, 185.
- Stevens, V.C. (1975) In: "Physiological Effects of Immunity Against Reproductive Hormones (eds, R.G. Edwards and M.H. Johnson) pp. 249. Cambridge University Press, London.
- Stevens, V.C. (1976) *Endocrinology*, 1, 379.
- Stevens, V.C., Powell, J.E., Lee, A.C. and David, G.D. (1981) *Fertil. Steril.* 36, 98.
- Stuart, M.C., Underwood, P.A., Harman, D.F., Payne, K.L., Rathjen, D.A., Razziuddin, S., Von Sturmer, S.R. and Vines, K. (1983) *J. Endocrinol.* 98, 323.
- Swaminathan, N. and Bahl, O.P. (1970) *Biochem. Biophys. Res. Commun.* 40, 422.
- Tandon, A., Das, C., Jailkhani, B.L. and Talwar, G.P. (1981) *Contraception* 24, 83.
- Tandon, A., Das, C., Jailkhani, B.L., Gaur, A., Sehgal, S., Gopinath, G. and Talwar, G.P. (1984) *J. Reprod. Fertil.* 70, 369.
- Tanford, C. (1968) In: "Advances in Protein Chemistry (eds., C.B. Anfinsen Jr., M.L. Anson, J.T. Edsall and F.M. Richards) Vol. XXIII, pp. 121, Academic Press, New York.
- Talib, S. (1975) Ph.D. Thesis, Aligarh Muslim University, Aligarh.

- Talwar, G.P., Dubey, S.K., Salahuddin, M. and Shastri, N. (1976a)
Contraception 13, 153.
- Talwar, G.P., Dubey, S.K., Salahuddin, M., Das, C., Hingorani, V.
and Kumar, S. (1976b) Contraception 13, 237.
- Thanwala, Y.M., Hay, F.C. and Stevens, V.C. (1978) Clin. Exp.
Immunol. 33, 403.
- Thanwala, Y.M., Hay, F.C. and Stevens, V.C. (1980) Clin. Exp.
Immunol. 39, 112.
- Thau, R.B., Yamamoto, Y., Goldstein, M., Ehrlich, P.H., Witkin,
S.S., Burrow, G.N., Canfield, R.E. and Bardin, C.W. (1983)
Contraception 27, 627.
- Tojo, S., Ashitaka, Y., Maruo, T. and Ohashi, M. (1977)
Endocrinol. Jpn. 24, 351.
- Tsong, Y.Y., Chang, C.C. and Nash, H.A. (1985) J. Reprod.
Immunol. 7, 139.
- Tullner, W.W. (1974) Contrib. Primatol. 3, 235.
- Van Hell, H.R., Matthijsen, R. and Homan, J.D.H. (1968) Acta
Endocr. Copenh. 59, 89.
- Vaitukaitis, J.L., Braunstein, G.D. and Ross, G.T. (1972)
Am. J. Obstet. Gynecol. 113, 751.
- Vaitukaitis, J.L. (1974) J. Clin. Endocr. Metab. 38, 755.

- Vaitukaitis, J.L., Ross, G.T., Braunstein, G.D. and Rayford, P.L.
(1976) Rec. Prog. Horm. Res. 32, 289.
- Veronese, F.M., Bevilacqua, R., Schiavon, O., and Rodighiero, G.
(1978) II. Farmaco. Ed. Sci. 33, 667.
- Veronese, F.M., Schiavon, O., Bevilacqua, R. and Rodighiero, G.
(1979) Z. Naturforsch. 34C, 392.
- Veronese, F.M., Bevilacqua, R., Schiavon, O. and Rodighiero, G.
(1979) II, Farmaco. Ed. Sci. 8, 716.
- Veronese, F.M., Schiavon, O., Bevilacqua, R., Bordin, F.
and Rodighiero, G. (1981) Photochem. Photobiol. 34,
351.
- Voller, A., Bidwell, D.E. and Bartlett, A. (1976) Bull. WHO
53, 55.
- Wada, H.C., Danisch, R.J., Baxter, S.R., Frederici, M.M.,
Fraser, R.C., Brownmiller, L.J. and Lankford, J.C. (1982)
Clin. Chem. 28, 1862.
- Whitaker, J.R. (1963) Anal. Chem. 35, 1950.
- Wide, L., Johannison, E., Tillingen, K.G. and Diczfalusy, E.
(1968) Acta Endocr. Copenh. 59, 579.
- Williams, A.F. (1977) In: "Contemporary Topics in Molecular
Immunology" (eds., R.R. Porter and G.L. Ada) Vol. 6,
pp. 83, Plenum Press, New York.

Wu, T., Ballon, S.C. and Teng, N.N. (1983) Am. J. Obstet.
Gynecol. 147, 821.

Yoshikawa, K., Mori, N. Sakakibara, S., Mizuno, N. and Song, P.S.
(1979) Photochem. Photobiol. 29, 1127.

Zondek, B. (1929) Endocrinologie 5, 425.